DEVELOPMENT AND CHARACTERIZATION OF HUMANIZED AND HUMAN FORMS OF ELR-CXC CHEMOKINE ANTAGONIST, BOVINE CXCL8$_{(3-74)}$K11R/G31P

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ABSTRACT

Glu-Leu-Arg (ELR)-CXC chemokine-mediated neutrophil migration and activation plays a key role in many inflammatory diseases. Dysregulated neutrophil activation often leads to inflammatory responses such as acute lung injury (ALI) or acute respiratory distress syndrome (ARDS).

Previously, we generated a bovine drug (i.e., bovine $CXCL8_{(3-74)}$K11R/G31P, bG31P) by mutating the first two amino acids at the beginning of the N-terminus of bovine CXCL8/IL-8 and later substituting Arg for Lys11 and Pro for Gly31. Bovine G31P was shown to be a highly effective ELR-CXC chemokine and neutrophil antagonist in cattle & guinea pigs, but a human equivalent thereof would be of significantly more use in human medicine. Published studies on the structure and function of human CXCL8 suggest that human $CXCL8_{(3-72)}$K11R/G31P (i.e., hG31P) would not be a particularly effective chemokine antagonist. Thus, development of a humanized form of bG31P became a primary goal. I first examined the effect of wholesale ligation of the carboxy half of hCXCL8 onto the amino half of bG31P and generated a human-bovine chimeric G31P (hbG31P; i.e., $bCXCL8_{(3-44)}$K11R/G31P-$hCXCL8_{(45-72)}$). I also made substitutions at each remaining human-discrepant amino acid (i.e., T3K, H13Y, T15K, E35A, and S37T) within the 5' half of the hbG31P cDNA. The results showed that hbG31P and its analogues blocked CXCL8-induced human neutrophil chemotactic responses, reactive oxygen intermediate (ROI) release, and intracellular calcium flux. Humanized bovine G31P was also shown to significantly block pulmonary neutrophilic pathology in a guinea pig model of airway endotoxemia.

As bG31P, hbG31P and its further humanized forms showed essentially equivalent ELR-CXC chemokine antagonist activity, Dr. Fang Li, Ms Jennifer Town and I then generated a fully human form of bG31P, hG31P. *In vitro*, hG31P was shown to effectively inhibit CXCL-1-, -5-, and -8-induced neutrophil chemotactic responses, intracellular Ca^{2+} flux, and ROI release. Human G31P also desensitized heterologous G protein-coupled receptors (GPCR) including bacterial peptides (e.g., N-formyl-methionine-leucine-

phenylalanine, fMLP), anaphylatoxin (e.g., complement 5a, C5a), lipid mediators (e.g., leukotriene B4, LTB4; platelet-activating factor, PAF) receptors. Moreover, hG31P, in a dose-dependent manner suppressed CXCL1 and CXCL8 expression by LPS-challenged airway epithelial cells and reversed the anti-apoptotic influence of ELR-CXC chemokines on neutrophils. *In vivo*, hG31P was significantly effective in blocking the pathology associated with airway endotoxemia, aspiration pneumonia, and intestinal ischemia and reperfusion injury, including neutrophil recruitment (70-95% reduction) into, and activation within, the airways or gut, chemokine or cytokine expression, and pulmonary vascular complications. The blockade of neutrophil recruitment by hG31P in aspiration pneumonia animals did not increase airway bacterial growth. The G31P treatment was protective in both mesenteric (i.e., local) and remote organ injury. These findings suggest that hG31P is not only a potent neutrophil antagonist, but an effective blocker of other inflammatory responses. These comprehensive anti-inflammatory effects indicate that hG31P could potentially provide a viable therapeutic approach for inflammatory diseases such as ALI /ARDS.

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Dedicated to

Baba, Mama for their love and support to my education

My wife Aimei and my son Daniel for their love and support

In memory of my grandmother for her love and care

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ABTS	2-2'-azino di-[3-ethylbenzthiazoline sulphonic acid]
AM	alveolar macrophage
ALI	acute lung injury
ANOVA	one-way analysis-of-variance
AP	aspiration pneumonia
AP-1	activator protein-1
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BLT	leukotriene B4 receptor
C5L2	complement C5a-like receptor 2
CFU	colony-forming unit
CINC	cytokine-induced neutrophil chemoattractant
COPD	chronic obstructive pulmonary disease
CSF	colony-stimulating factor
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECP	eosinophil cationic protein
ELR	Glu-Leu-Arg
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial neutrophil-activating peptide-78
EPO	eosinophil peroxidase

ERK	extracellular signal-regulated kinase
ESAM	endothelial cell-selective adhesion molecule
E-selectin	endothelial selectin
FBS	fetal bovine serum
fMLP	formyl-methionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
FPRL1	FPR-like protein 1
GCP-2	granulocyte chemotactic protein-2
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GRO	growth related oncogene
GST	glutathione transferase
HBSS	Hank´s balanced salt solution
H&E	hemotoxylin and eosin
HIV-1	human immunodeficiency virus 1
HTAB	hexadecyltrimethylammonium bromide
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule 1
IPTG	isopropyl-thio-D-galactopyranoside
IBD	inflammatory bowel disease
IL	interleukin
IR	ischemia and reperfusion injury
JAM	junctional adhesion molecule

KC	keratinocyte-derived cytokine
LF	lactoferrin
LFA-1	lymphocyte function–associated antigen 1
LO	lipoxygenase
LPS	lipopolysaccharide
L-selectin	leukocyte selectin
LT	leukotriene
LX	lipoxin
MBP	major basic protein
MCP	monocyte chemoattractant protein
MEM	minimum essential medium
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
NAP-2	neutrophil-activating peptide-2
NF-κB	nuclear factor-κB
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PECAM	platelet/endothelial-cell adhesion molecule
PF4	platelet factor 4
PG	prostaglandin
PLA_2	phospholipase A_2
PMN	polymorphonuclear cell (ie, neutrophil)

PMSF	phenylmethysufonyl fluoride
P-selectin	platelet selectin
PSGL	platelet selectin glycoprotein ligand
qRT-PCR	quantitative real time PCR
RA	rheumatoid arthritis
RANTES	regulated upon activation, normal T cell expressed and secreted
RBC	red blood cells
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
SMA	superior mesenteric artery
sTNFR	soluble TNF receptors
TNF	tumor necrosis factor
TMB	tetramethylbenzidine
VCAM-1	vascular cell adhesion molecule 1
WBC	white blood cells

CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Inflammation is a protective immune response by the organism to remove harmful stimuli such as pathogens, damaged cells, or irritants. It is characterized by the exudation and extravasation of plasma and leukocytes respectively from vascular tissues into injured tissues. Even though this normal physiological response is designed to fight infection, remove damaged cells, and stimulate healing, the excessive recruitment of leukocytes often exacerbates tissue damage, slows healing, and in some cases leads to host death (Williams, Kus et al. 1994). Among these cells, neutrophils are considered major effector in acute inflammatory diseases, such as acute lung inury (ALI) and aspiration pneumonia (AP) (Sibille and Marchandise 1993; Abraham, Carmody et al. 2000). They are also important in autoimmune diseases (e.g., rheumatoid arthritis, RA), chronic inflammatory diseases (e.g., inflammatory bowel diseases, IBD), and non-infectious diseases (e.g., ischemia-reperfusion [I/R] injury) (Johnston, Burns et al. 1999; Lefkowitz and Lefkowitz 2001; Kalia, Brown et al. 2003).

There are many mediators that are involved in mediating neutrophil recruitment, such as bacterial peptides (e.g., N-formyl-methionine-leucine-phenylalanine, fMLP), anaphylatoxin (e.g., complement factor 5a, C5a), lipid mediators (e.g., leukotriene B4, LTB4; platelet-activating factor, PAF), and Glu-Leu-Arg (ELR)-CXC chemokines (e.g., interleukin-8, IL-8) (Bless, Warner et al. 1999; Crooks, Bayley et al. 2000). Among these mediators the ELR-CXC chemokines (e.g., CXCL8/IL-8) have been considered to be particularly important in many settings (Harada, Mukaida et al. 1996; Baggiolini 1998; Shames, Zallen et al. 2000). This group of CXC chemokines bind to either the CXCR1 or CXCR2 receptors (Loetscher, Seitz et al. 1994; Ahuja and Murphy 1996; Baggiolini 1998; Richardson, Pridgen et al. 1998; Wuyts, Proost et al. 1998; McColl and Clark Lewis 1999) and thereby induce largely overlapping effects. Their importance in neutrophilic

inflammation suggests that development of an antagonist should be a research priority (Harada, Mukaida et al. 1996; Baggiolini 1998; Shames, Zallen et al. 2000).

To ameliorate the pathology induced by overly exuberant neutrophil activation and recruitment, many therapeutic tools have been developed in the past few years. Steroids, such as dexamethasone, are widely used anti-inflammatory drugs used for treating inflammatory and autoimmune diseases such as RA and lupus. Although effective, steroid use often has many side effects such as stomach upset, diabetes and immunosuppressant actions. Since proinflammatory cytokines such as TNFα and IL-1β are extremely important in mediating inflammatory diseases, the antagonists of these cytokines and their receptors have been extensively examined and some of these are already being used clinically (van Deventer 1999). In order to block neutrophil-mediated inflammatory disease, research has also targeted neutrophil chemoattractants such as C5a, LTB4, and PAF with the goal of developing antagonists for these important mediators. They are found to be able to prevent C5a-induced injury during sepsis, ameliorate I/R-induced neutrophil-mediated tissue injury, or reverse arthritis pathology (Karasawa, Guo et al. 1991; Huber-Lang, Riedeman et al. 2002; Moreno, Alves-Filho et al. 2006; Diaz-Gonzalez, Alten et al. 2007). Targeted to inhibit the function of CXCL8 in inflammatory diseases, a CXCL8 monoclonal antibody was developed and found to be able to prevent neutrophil infiltration and tissue injury (Sekido, Mukaida et al. 1993). Other CXCL8 receptor antagonists such as SB-26510, a non-peptide CXCR2 antagonist, and repertaxin, a CXCR1/R2 receptor antagonist, have recently been developed to inhibit neutrophil-mediated pathology (Auten, Richardson et al. 2001; Casilli, Bianchini et al. 2005).

Our laboratory had engineered a high affinity analogue of bovine CXCL8, $CXCL8_{(3-74)}$ K11R (Li and Gordon 2001), from which we have generated a very high affinity antagonist, $CXCL8_{(3-74)}$K11R/G31P (Li and Gordon 2002) (hereafter referred to as 'bG31P'). bG31P binds to bovine neutrophils via both the CXCR1 and CXCR2 and thereby blocks the chemotactic activities of multiple ELR-CXC chemokines. Very low doses of bG31P block all of the chemotactic activities present in extracts from bovine pneumonic pasteurellosis or endotoxin-induced mastitis lesions, as well as neutrophil infiltration of intradermal endotoxin-challenge sites in cattle (Li, Zhang et al. 2002). Since bovine and human CXCL8 share relatively high amino acid sequence identity, with most

of the discrepant amino acids residing in the carboxyl half of the molecule (Tabel 3.1) (Morsey, Popowych et al. 1996), I hypothesized that simply replacing the bovine carboxyl half of bG31P with that of human CXCL8 would make a similarly effective ELR-CXC chemokine antagonist (the amino terminal half of bG31P contained all of the previously introduced substitutions). In Chapter 3 of my thesis, I developed a humanized form of bG31P through wholesale ligation of the carboxy half of human CXCL8 onto the amino half of bG31P and characterized its activity through *in vitro* and *in vivo* experiments. I demonstrated that this human-bovine chimeric G31P (hbG31P; i.e., $bCXCL8_{(3-44)}$K11R/G31P-$hCXCL8_{(45-72)}$) fully retained the ELR-CXC chemokine antagonist activity of bG31P. Meanwhile, our lab had created a fully human form of bG31P (hG31P; i.e., $CXCL8_{(3-72)}$ K11R/G31P) which has also shown effective antagonist activity in preliminary *in vitro* experiments. Thereafter, I carried on characterizing this hG31P's antagonist activity in *in vitro* experiments and several *in vivo* model systems including airway endotoxemia and aspiration pneumonia in guinea pigs, and superior mesenteric artery ischemia and reperfusion injury in rats (Chapter 4, 5, 6). The remainder of this chapter (Chapter 1) and next chapter (Chapter 2) are the project literature review, research hypothesis and objectives.

1.2. LITERATURE REVIEW

1.2.1. Proinflammatory inflammatory mediators

1.2.1.1. Cytokines

Proinflammatory cytokines are a group of cytokines that function as amplifiers of inflammatory reactions. These include TNFα, IL-1β, and IL-6 (Malaviya 2006). Among these cytokines, TNFα and IL-1β are the so-called "early response cytokines" because of their roles in initiating and furthering inflammatory responses (Dinarello 2000). They also serve to regulate other cellular functions and tissue repair in cases of local inflammation (Le and Vilcek 1987). However, excessive release of these cytokines can result in tissue injury and even host death (Abraham, Carmody et al. 2000).

TNFα

TNFα is produced by many different cell types such as monocytes/macrophages (Ulich, Watson et al. 1991; Beck-Schimmer, Schwendener et al. 2005), endothelial cells (Ranta, Orpana et al. 1999), smooth muscle cells (Newman, Zhang et al. 1996), and neutrophils (Lapinet, Scapini et al. 2000; Sohn, Paape et al. 2007). Brochial fibroblasts and epithelial cells from cancer patients in coculture, but not in monoculture, also release TNFα (~ 100pg/ml). But the main source of TNFα *in vivo* is stimulated-monocytes/macrophages, especially in endotoxin-induced lung injury (Beck-Schimmer, Schwendener et al. 2005). TNFα is multifunctional. Like IL-1β, it is an endogenous pyrogen. Both can induce fever after being injected intravenously (Dinarello, Cannon et al. 1986). TNFα also stimulates macrophages (Strieter, Chensue et al. 1990; Ciesielski, Andreakos et al. 2002), endothelial cells (Smart and Casale 1994), and pulmonary epithelial cells (Allen, Menendez et al. 2000) to release chemokines such as CXCL8, growth-related oncogene (GRO) α and epithelial neutrophil-activating peptide (ENA)-78. These ELR-chemokines along with TNFα are important neutrophil chemotactic factors in inflammation (Ming, Bersani et al. 1987; Rekdal, Konopski et al. 1994). TNFα also stimulates endothelial cells to produce PAF (Bussolino, Camussi et al. 1988) and express adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (McHale, Harari et al. 1999). These findings indicate that TNFα is a very important inflammatory mediator and plays critical roles in mediating neutrophil recruitment to inflammation sites, both directly and indirectly.

In addition to its role in facilitating neutrophil transendothelial migration (Bussolino, Camussi et al. 1988; McHale, Harari et al. 1999), TNFα also stimulates endothelial cells and neutrophils to generate large amounts of reactive oxygen intermediates (ROI) (reviewed in (Mukhopadhyay, Hoidal et al. 2006)), which are involved in TNFα-induced endothelial cell apoptosis. Apoptotic vascular endothelial cells express procoagulant activities and thereby may be associated with atherosclerosis (Bombeli, Karsan et al. 1997; Deshpande, Angkeow et al. 2000). TNFα also plays an important role in intestinal I/R injury. Elevated local and systemic TNFα levels can be found in intestinal I/R injury models, where its production facilitates neutrophil influx and contributes to tissue injury (Souza, Cassali et al. 2001). TNFα inhibition studies showed

that it is also highly associated with ischemia reperfusion injury-induced lethality (Souza, Cassali et al. 2001; Souza, Pinho et al. 2003). Moreover, it is indicated that TNFα plays a major role in RA and Crohn's diseases (van Deventer 1999; Edrees, Misra et al. 2005).

Strategies to inhibit TNF function, such as monoclonal anti-TNF antibodies and soluble TNF receptors (sTNF-R), are widely studied and used for treating RA, Crohn's disease and other dieases (van Deventer 1999; Kadokami, McTiernan et al. 2001; Bartolucci, Ramanoelina et al. 2002; Edrees, Misra et al. 2005). However, the potential side effects of long-term use of anti-TNF treatment include the risk of serious infections and malignancies (Costenbader, Glass et al. 2006).

IL-1β

IL-1β is another important proinflammatory cytokine. IL-1β can be produced by monocytes/macrophages (Arend, Gordon et al. 1989; Ulich, Watson et al. 1991), neutrophils (Parsey, Tuder et al. 1998) and endothelial cells (Nawroth, Bank et al. 1986) when stimulated by LPS or TNFα. It is also found to be expressed in intestinal epithelial cells following challenge with LPS *in vitro* (Waterhouse and Stadnyk 1999). But the main source of IL-1β is considered to be neutrophils, especially in hemorrhagic or endotoxemic lung tissue (Parsey, Tuder et al. 1998). IL-1β has functions similar to TNFα such as induction of fever, chemokine production (e.g., IL-8, macrophage inflammatory protein [MIP]-2) by alveolar macrophages and pulmonary epithelial cells (Strieter, Chensue et al. 1990; Ishii, Fujii et al. 2004; Manzer, Wang et al. 2006), and expression of adhesion molecules (e.g., ICAM-1 and VCAM-1) by endothelial cells (McHale, Harari et al. 1999). In general, IL-1β also plays a crucial role in initiating inflammatory responses and in recruitment and activation neutrophils to inflammation sites.

Studies show that intratracheal instillation of IL-1β induces more acute lung injury than does TNFα (Ulich, Watson et al. 1991). And clinically, IL-1β, rather than TNFα, is found to be a major inflammatory cytokine and associated with higher risk of death in ARDS patients (Goodman, Strieter et al. 1996; Pugin, Ricou et al. 1996). IL-1β is also involved in ischemia and reperfusion injury, but its precise role is still uncertain. Some studies using IL-1 receptor antagonist (IL-1ra) and IL-1 inhibitor have demonstrated that

IL-1β plays deleterious roles in renal and intestinal I/R injuries, repectively (Haq, Norman et al. 1998; Yamamoto, Tanabe et al. 2001). Other studies have mentioned that inhibition of IL-1 by use of anti-IL-1 antiserum or IL-1ra exacerbated the I/R tissue injury (e.g., increasing vascular permeability), TNFα expression, and lethality (Souza, Guabiraba et al. 2003).

IL-6

IL-6 is a pleiotropic cytokine produced during inflammatory responses. It can be produced by a variety of cells such as alveolar macrophages, monocytes, fibroblasts, epithelial cells and other cells. The IL-6 inducers include bacterial products (e.g., LPS) or cytokines (e.g., TNFα, IL-1β) (Barton 1996; Larsson, Larsson et al. 1999). IL-6 is considered to be a prototypic proinflammatory cytokine because of its high production after LPS exposure (Barton 1996; Tominaga, Kirikae et al. 1997). In acute inflammatory responses, it induces fever and increases acute phase protein expression by the liver (Barton 1996). IL-6, combined with sIL-6R is also involved in neutrophil recruitment in local inflammation by inducing CXCL8 and adhesion molecule production by endothelial cells (Romano, Sironi et al. 1997). The induction of chemokine expression (e.g., macrophage inflammatory protein-2, MIP-2) by IL-6 is also found in virus (e.g., Herpes simplex virus -1, HSV-1)-induced inflammation (Fenton, Molesworth-Kenyon et al. 2002). IL-6 has been demonstrated to significantly contribute to lung injury and neutrophil recruitment in a rat hemorrhagic shock model (Hierholzer, Kalff et al. 1998). It has also been shown that IL-6 plays an important role in mediating lung leakage, neutrophil infiltration, and chemokine expression in lung injury following kidney I/R (Klein, Hoke et al. 2008).

Recently, it is believed that IL-6 also plays an important role in regulating inflammation. For instance, IL-6 can inhibit TNFα production induced by endotoxin challenge in human and mouse model systems (Aderka, Le et al. 1989; Starkie, Ostrowski et al. 2003). Intratracheal instillation of IL-6 leads to dramatic reductions in vascular permeability and neutrophil sequestration in the lung, and in the levels of TNFα and MIP-2

expression in bronchoalveolar lavage (BAL) fluids in an immunoglobulin G immune complex-induced lung injury model (Shanley, Foreback et al. 1997).

1.2.1.2. Complement

The complement system includes some 35 linked soluble plasma proteins and receptors (Sunyer, Boshra et al. 2005). The complement cascade can be activated by classical, alternative or lectin pathways, which are initiated by antigen-antibody complexes, various types of microbial surface molecules (e.g., LPS), or microorganisms containing mannans on their surfaces, respectively (Guo and Ward 2005; Sunyer, Boshra et al. 2005). Upon initiation, complement cascade products play crucial roles in the phagocytosis of invading microorganisms, solubilization of immune complexes, and initiation of inflammatory responses (Sunyer, Boshra et al. 2005). However, complement activation has also been demonstrated to be important in the pathogenesis of many inflammatory diseases, such as sepsis (Czermak, Sarma et al. 1999), acute respiratory distress syndrome (ARDS) (Stevens, O'Hanley et al. 1986), RA (Grant, Picarella et al. 2002), IBD (Woodruff, Arumugam et al. 2003), and I/R injury (Riedemann and Ward 2003). The harmful roles that complement activation exerts are prominent in promoting and perpetuating inflammatory reactions (Guo and Ward 2005).

Among the complement protein split products, C5a is one of the most potent peptides. C5a is a powerful chemoattractant for neutrophils, monocytes, and macrophages and can also induce the respiratory burst and release of granule enzymes in neutrophils and facilitate their phagocytotic processes (Guo and Ward 2005). C5a can modulate cytokine expression by inflamed respiratory epithelial cells and microvascular endothelial cells (Laudes, Chu et al. 2002; Riedemann, Guo et al. 2002). It can stimulate neutrophils and enhance their expression of adhesion molecules such as CD11b/CD18 (Foreman, Glovsky et al. 1996), as well as delay neutrophil apoptosis. It also functions as an important vasodilator during inflammation (Guo and Ward 2005). C5a binds two kinds of receptors on neutrophils, the C5aR and the C5L2 (Sengelov 1995; Ohno, Hirata et al. 2000). C5aR is a G protein-coupled receptor (GPCR) with seven transmembrane segments. It is related to most of the proinflammatory functions of C5a in inflammatory diseases (Grant, Picarella

et al. 2002; Riedemann and Ward 2003; Woodruff, Arumugam et al. 2003). C5L2 is similar to C5aR and has been recently reported to be a critical regulator involved in C5a and LPS signaling (Chen, Mirtsos et al. 2007). Since C5a is a GPCR, as are the receptors for other neutrophil chemoattractants such as CXCL8, fMLP, LTB4, and PAF, cross-desensitization of their individual receptors has been carefully studied. There is evidence that fMLP, C5a, and CXCL8 desensitize each others' Ca^{2+}-mobilizing responses; PAF and LTB4 were also desensitized by peptide chemoattractants, but not *vice versa* (Didsbury, Uhing et al. 1991; Tomhave, Richardson et al. 1994; Richardson, Ali et al. 1995). This evidence affords the possiblity of studying the desensitization of heterologus chemoattractant receptors by chemokine receptor antagonists.

As an important proinflammatory mediator, C5a plays key roles in exacerbating the pathology in inflammatory diseases through facilitating neutrophil recruitment and activation and inducing other inflammatory mediators' release. For instance, C5a is known to stimulate pulmonary macrophages to produce proinflammatory cytokines (e.g., TNFα and IL-1β), which cause up-regulation of vascular ICAM-1 and E-selectin on vascular endothelial cells (Mulligan, Schmid et al. 1996). It interacts with endothelial cells through binding to the C5a receptor to induce P-selectin expression (Ward 1996). It also up-regulates β2-integrin expression and induces shedding of L-selectin on neutrophils (Jagels, Daffern et al. 2000). C5a also can stimulate neutrophils to release leukotrienes (including LTC4 and LTD4), and histamine to increase vascular permeability and slow blood flow (Markiewski and Lambris 2007). In addition, C5a is an important neutrophil chemoattractant and forms a gradient to induce PMN motility in tissue. C5a is also able to stimulate endothelial cells, epithelial cells, as well as alveolar macrophages to produce IL-8 and indirectly contributes to neutrophil migration to inflammation sites (Markiewski and Lambris 2007). C5a is reported to be able to bind C5aR on rat alveolar epithelial cells and stimulate them to release TNFα and IL-1β, which further excerbates inflammatory responses (Riedemann, Guo et al. 2002).

In specific inflammatory disease models (e.g., I/R injury and aspiration pneumonia), the roles of C5a have been widely investigated. In hindlimb I/R injury, neutralizing C5a antibody treatment indicates that C5a is involved in pulmonary neutrophil recruitment, tissue CXC chemokine expression, and vascular permeability changes. But blockade of

C5a seems to be less effective than ELR-CXC chemokine inhibition (e.g., with anti-CINC and anti-MIP-2 antibodies) in ameolirating pulmary neutrophil recruitment and vascular permeability (Bless, Warner et al. 1999). This finding indicates that ELR-CXC chemokines may play more important roles in the aforementioned pathological changes. In aspiration pneumonia, C5a contributes to the accumulation of both neutrophils and alveolar macrophages (Ishii, Kobayashi et al. 1989). Interestingly, C5a is found not important for pulmonary neutrophil sequestration in endotoxin-induced lung injury (Rittirsch, Flierl et al. 2008).

1.2.1.3. Lipid mediators

Lipid mediators are derived from arachidonic acid (AA), a key lipid mediator released from the phospholipid cell membrane upon the action of phospholipase A_2 (PLA_2) during inflammation. There is now considerable evidence to suggest that metabolites of the AA cascade, namely prostanoids, leukotrienes and lipoxins, play a critical role during inflammation. AA can be metabolized by three pathways, including the cyclooxygenase, lipoxygenase (LO), and lipoxin cell-cell interaction pathways which lead to prostaglandin (PG), leukotriene (LT), and lipoxin (LX) production, respectively. LTs and PGs amplify acute inflammation, whereas LXs have unique anti-inflammatory activities (Levy, Clish et al. 2001).

LTB4

Leukotriene B4 (LTB_4) is a 5-LO-derived potent neutrophil chemoattractant. It can be produced by neutrophils, mast cells, eosinophils, and macrophages stimulated by proinflammatory cytokines such as TNFα, IL-1β and chemoattractants such as CXCL8, C5a, PAF, and itself (Crooks and Stockley 1998; Serhan 2000). In addition to its chemotactic activity, it can cause neutrophil aggregation, calcium mobilization, and release of granule products and superoxide anions (Levy, Clish et al. 2001). Leukotrienes exert their effects through binding to two kinds of GPCRs, the BLT_1 and the BLT_2. The BLT_1, a high-affinity receptor for LTB_4 expressed on leukocytes, mast cells, and endothelial cells,

mediates most LTB_4-dependent inflammatory diseases such as asthma, RA, and arteriosclerosis. BLT_2 is a lower-affinity receptor for LTB_4 (Lundeen, Sun et al. 2006; Qiu, Johansson et al. 2006; Shao, Del Prete et al. 2006) and its function is still not clear.

LTB_4 is well known to be significantly involved in neutrophil recruitment and activation during inflammatory responses. But, the mechanism by which LTB_4 recruits PMN is somewhat different from that of fMLP, CXCL8 and C5a. Studies using a LTB_4 receptor antagonist demonstrates that LTB_4 plays a more important role than fMLP in neutrophil transendothelial migration, although it has a lower capability to chemoattract neutrophils than fMLP (Nohgawa, Sasada et al. 1997). Antibody blockade experiments indicate that ICAM-1 participates in LTB_4-induced neutrophil transendothelial migration, but LTB_4 does not directly induce ICAM-1 expression (Nohgawa, Sasada et al. 1997; Crooks and Stockley 1998). LTB_4 increases the expression of β2-integrin on neutrophils, which can facilitate neutrophil adherence and migration (Crooks and Stockley 1998), even though LTB_4 and CXCL8 are reported to be able to stimulate β2-integrin-independent migration of PMN across human pulmonary endothelial cells *in vitro* (Mackarel, Russell et al. 2000). LTB_4 does not stimulate endothelial cells to produce CXCL8 or PAF (McIntyre, Zimmerman et al. 1986; Nohgawa, Sasada et al. 1997). In addition, *in vitro* experiments indicate that human umbilical vein endothelial cells (HUVEC) express LTB_4 receptors (BLT_1 and BLT_2), which are more highly expressed after LPS and TNFα stimulation and more responsive to LTB_4. LTB_4 can augment nitric oxide and MCP-1 production from HUVEC stimulated by LPS (Qiu, Johansson et al. 2006). This is an important feature of LTB_4, because MCP-1 facilitates PMN transmigration across endothelial cells barriers (Maus, Waelsch et al. 2003). This data suggests that LTB_4 interaction with LPS (or TNFα)-stimulated endothelial cells through the BLT_1 and the BLT_2 can enhance PMN transmigration across endothelial cells. LTB_4 certainly forms a chemotactic factor gradient to attract PMN migration to the tissue. LTB_4 facilitates PMN transepithelial cell migration through the ROS-extracellular signal-regulated kinase-linked cascade (Woo, Yoo et al. 2003).

PAF

Platelet-activating factor (PAF) is a member of a family of structurally-related phospholipids with various biological activities, including activation of platelets, leukocytes and endothelial cells (Soares, Pinho et al. 2002). It is produced by a variety of cells that participate in the inflammatory responses, including neutrophils, platelets, mast cells, monocytes/macrophages, and endothelial cells. There are several important mediators that induce endothelial cells to produce PAF, such as thrombin, histamine, IL-8, TNFα and IL-1α (Montrucchio, Alloatti et al. 2000). PAF targets these and other cells via a specific GPCR (i.e. PAFR).

PAF is a potent neutrophil chemoattractant, but also participates in neutrophil migration and activation in other ways, such as by activating endothelial cells. Thrombin-challenged endothelial cells secrete PAF, which stays on the surface of endothelial cells, and thereby can induce PMN adhesion via the neutrophils PAFR (Zimmerman, McIntyre et al. 1990). The signaling component provided by endothelial cell-associated PAF may facilitate activation and polarization of the β2-intergrins on neutrophils and contribute to PMN rolling and firm adhesion (Lorant, Patel et al. 1991; Macconi, Foppolo et al. 1995). PAF also participates in the adhesion of PMNs to, and migration across, TNFα-stimulated HUVEC, but not in cell rolling (Macconi, Foppolo et al. 1995). Endothelial cell-associated PAF co-stimulates TNFα-induced neutrophil respiratory burst activity during TNFα-stimulated endothelial-neutrophil interactions (von Asmuth and Buurman 1995). PAF may also promote TNFα-induced ICAM-1 and E-selectin expression on endothelial cells, thereby facilitating neutrophil adherence to the endothelium (Sterner-Kock, Braun et al. 1996). It also can activate neutrophils to produce elastase and thereby facilitate neutrophil transendothelial migration (Cepinskas, Sandig et al. 1999). After neutrophils transmigrate across the endothelium, PAF also contributes to neutrophil adherence to fibroblasts (Burns, Simon et al. 1996). PAF also works as a potent chemoattractant for neutrophils and stimulates lysosomal enzyme release and reactive oxygen product formation by neutrophils, eosinophils, and macrophages (Lad, Olson et al. 1985).

1.2.1.4. Bacterial products

fMLP

The bacterial tripeptide formyl-Met-Leu-Phe (fMLP) has been discovered as the major peptide neutrophil chemotactic factor produced by *Escherichia coli* (Marasco, Phan et al. 1984). In addition to chemotaxis, fMLP also plays an important role in inducing neutrophil superoxide release (e.g., O^{2-}), proteolytic enzyme (e.g., lysosomal enzyme) release, degranulation (e.g., myeloperoxidase [MPO] release), metabolism of arachidonic acid, and calcium mobilization (Naccache, Showell et al. 1977; Hatch, Gardner et al. 1978; Bokoch and Reed 1980). fMLP binds to the high-affinity formyl peptide receptor (FPR) and the low-affinity FPR-like-1 protein (FPRL1) on neutrophils (Selvatici, Falzarano et al. 2006). These two receptors are both GPCRs and share 69% homology at the amino acid level. Compared to FPRL1, FPR plays a more important role in mediating neutrophil chemotaxis and Ca^{2+} mobilization. In addition to the expression on the neutrophil, both receptors are expressed on monocytes, hepatocytes, and other resident tissue cells. FPRL1 is also expressed on epithelial cells and microvascular endothelial cells (Le, Yang et al. 2002). FPRL1 expression on monocytes and microglia may be involved in Alzheimer's disease (Cui, Le et al. 2002). Since fMLP, along with C5a, LTB4, PAF, and IL-8, binds GPCRs on neutrophils, their roles in mediating neutrophil signaling have been widely studied. *In vitro*, they all undergo homologous desensitization of neutrophil Ca^{2+} mobilization. Neutrophil peptide ligands (C5a, fMLP, and IL-8) can heterologously desensitize lipid ligand (PAF and LTB4) receptors. Among peptide ligands, they can differentially desensitize each others ligand receptors, and fMLP is highly effective in desensitizing other peptide ligand receptors (Tomhave, Richardson et al. 1994). *In vivo*, fMLP and chemokines (e.g., MIP-2, keratinocyte-derived cytokine [KC]) contribute differentially to neutrophil migration and activation in response to increasing levels of bacterial challenge and at different stages after challenge in pneumococcal pneumonia. fMLP seems to be important in situations with high bacterial inoculum and induces neutrophil migration and activation during the first 12 hours after bacterial challenge, whereas chemokines are important in low bacterial inoculum situations and contribute to a more sustained neutrophil response (Gauthier, Fortin et al. 2007).

LPS

Lipopolysaccharide (LPS), the endotoxin of gram-negative bacteria, is another important bacterial product that induces a variety of inflammatory responses. It binds to LPS-binding proteins (LBP) in plasma and forms LPS-LBP complex, which further interacts with cluster of designation-14 (CD14) and toll-like receptor 4 (TLR4) and leads to activation of TLR4 on the cells (Miller, Ernst et al. 2005). In the lung, TLR4 can be expressed on alveolar macrophages (Hollingsworth, Chen et al. 2005), endothelial cells (Andonegui, Bonder et al. 2003), and epithelial cells (Armstrong, Medford et al. 2004; Guillot, Medjane et al. 2004). It also can be expressed on hematopoietic cells such as neutrophils (Sabroe, Jones et al. 2002; Sabroe, Prince et al. 2003) and monocytes (Sabroe, Jones et al. 2002).

When the airway is exposed to LPS challenge, airway epithelial cells are the primary responders to the challenge. When challenged with LPS, pulmonary epithelial cells, including tracheobronchial epithelium and alveolar epithelium, release ELR-CXC chemokines (e.g., IL-8, KC), cytokines (e.g., IL-6), and defensins (e.g., β defensin-2) (Becker, Diamond et al. 2000; Guillot, Medjane et al. 2004). The production of these inflammatory mediators is dependent on TLR4 and CD14. The alveolar macrophage is another important cell type involved in airway endotoxemia. It plays a major role in producing proinflammatory cytokines (e.g., TNF, IL-1, and IL-6) and chemokines (e.g., IL-8, MCP-1) (Larsson, Larsson et al. 1999; Thorley, Ford et al. 2007). These macrophage-derived mediators further stimulate alveolar epithelial cells to amplify production of these mediators and thereby exacerbate the LPS-induced inflammatory responses (Thorley, Ford et al. 2007). Like airway epithelial cells, TLR4 is also significantly involved in macrophage activation and its functional roles. Studies have demonstrated that TLR4 expressed on macrophages is more important than that on other resident cells (e.g., epithelial cell, endothelial cell) in neutrophil recruitment during LPS-induced airway inflammation (Hollingsworth, Chen et al. 2005). In addition to inducing epithelial cells to generate cytokines and facilitating neutrophil infiltration, LPS challenge also causes damage to airway epithelial cells, such as increasing epithelial permeability (Eutamene, Theodorou et al. 2005).

Endothelial cell-expressed TLR4 is also involved in LPS-induced pulmonary neutrophil sequestration into the lung following both intraperitoneal and airway challenge (Andonegui, Bonder et al. 2003; Hollingsworth, Chen et al. 2005). As discussed above, endothelial TLR4 is less important than macrophage TLR4 in airway endotoxemia-induced neutrophil infiltration (Hollingsworth, Chen et al. 2005). But, it is particularly important in systemic endoxemia-induced pulmonary neutrophil infiltration (Andonegui, Bonder et al. 2003). One reason seems to be the increased expression of P-selectin and VCAM-1 by LPS-activated endothelial cells and their expression is TLR4-dependent (Andonegui, Goyert et al. 2002). LPS challenge also causes pulmonary microvascular endothelial cell leakage, which is an important part of the pathophysiology of human lung injury (Shelton, Wang et al. 2006).

LPS is also able to activate neutrophils, causing expression of β2-integrin, increased L-selectin shedding, respiratory burst, cytokine (e.g., TNFα, IL-β), and chemokine (e.g., IL-8) generation (Sabroe, Jones et al. 2002; Sabroe, Prince et al. 2003). Neutrophils also express TLR4 and CD14. During LPS challenge in the lung, TLR4, CD14, and LBP are all significantly involved in neutrophil activation (Jack, Fan et al. 1997; Sabroe, Jones et al. 2002). Notably, LPS activation of neutrophils through TLR4 can down-regulate ELR-CXC chemokine receptor (CXCR1 and CXCR2), expression by neutrophils, although CXCR1 expression is more resistant to such down-regulation (Sabroe, Jones et al. 2005). This process may be caused by the role of TLR4 in down-regulating G-protein receptor kinases. Oddly, the down-regulation of CXCR1/2 expression does not reduce, but rather, it enhances neutrophil responses to chemokines (Fan and Malik 2003; Sabroe, Jones et al. 2005).

In summary, both systemic and airway administration of LPS can induce pulmonary neutrophil recruitment which is TLR4-dependent. LPS, given through airway, can cause the recruitment and extravasation of neutrophils, injury to the alveolar epithelium and disruption of pulmonary capillary integrity, leading to a protein-rich fluid leakage into the alveolar space.

1.2.1.5. Chemokines

Chemokines are a family of small proteins (8-10 kDa) that have a common role in regulating leukocyte migration in normal or inflamed tissues. However, the first member of the chemokine family, platelet factor 4 (PF4) (Poplawsky and Niewiarowaki 1964), was not recognized as a chemotactic cytokine until after IL-8 was discovered and characterized as a neutrophil chemotactic factor in 1987 (Yoshimura, Matsushima et al. 1987). Chemokines have conserved cysteine residues such that, based on their relative positioning, they are divided into four subgroups (CXC, CC, CX_3C and C). CXC, CC, and CX3C chemokines have four to six cysteines, and C chemokines only have two. To date, approximately 50 chemokines have been discovered (Allen, Crown et al. 2007). In the new systematic chemokine nomenclature, chemokines are named based on their cysteine subclass roots, followed by "L" for ligand. The assigned numbers correspond generally to that used in the corresponding gene nomenclature. For example, IL-8 is now called CXCL8 (Zlotnik and Yoshie 2000). Chemokine receptors consist of a large group of the rhodopsin family of cell-surface G protein-coupled receptors with seven-transmembrane domains. Chemokines function through binding to these receptors on the surface of target cells. The nomenclature system of chemokine receptors follows the chemokine subclass specificity and followed by "R". For example, the two CXCL8 receptors are CXCR1 and CXCR2 (Mukaida 2003).

Chemokines have diverse roles in innate and adaptive immunity. In addition to their roles in leukocyte migration, which include integrin activation during leukocyte-endothelial interactions and leukocyte chemotaxis, chemokines are also involved in leukocyte degranulation and promotion of angiogenesis or angiostasis (Mackay 2001; Mukaida 2003). It is also believed that chemokines contribute to dendritic cell function, T cell differentiation and function, inflammatory diseases (e.g., rheumatoid arthritis and multiple sclerosis), mucosal and subcutaneous immunity, and subversion of immune responses by viruses (e.g., HIV-1) (Mackay 2001). Further discussion of the ELR subclass of chemokines is presented as below (§1.2.3.)

1.2.2. Inflammatory cells

1.2.2.1. Neutrophils

1.2.2.1.1. General aspects of neutrophils

Neutrophils play a critical role in fighting microorganisms, e.g., bacteria, fungi, and viruses in host innate immunity (Smith 1994). Neutrophils can be activated by inflammatory mediators such as cytokines, complement components, arachidonic acid metabolites, or chemokines (Baggiolini 1995). Following the gradients of chemoattractants, activated neutrophils migrate into infection sites and their release inflammatory mediators and antimicrobial products that contribute to pathogen clearance (Pettersen and Adler 2002). However, excessive or inappropriate neutrophil activation can cause tissue damage or even host death (Smith 1994). Many inflammatory diseases are exacerbated by neutrophil activation, such as ALI, AP (Sibille and Marchandise 1993; Abraham, Carmody et al. 2000), I/R injury (Kalia, Brown et al. 2003), chronic obstructive pulmonary diseases (COPD) (Stockley 2002), and ARDS (Lee and Downey 2001). Recently, neutrophils were also found to be involved in initiating and modulating adaptive immune responses, such as inducing maturation of dendritic cells (DC), which can trigger strong T cell proliferation (van Gisbergen, Sanchez-Hernandez et al. 2005).

1.2.2.1.2. Neutrophil development

Neutrophils develop from $CD34^+$ progenitor cells in the bone marrow. In this process, myeloid precursors undergo striking morphologic and functional changes characterized by significant alterations in cell size and nuclear shape (Lichtman and Weed 1972; Lund-Johansen and Terstappen 1993). They also develop stage-specific organelles containing proteins necessary for the highly specialized roles of neutrophils in phagocytosis, bacterial killing, and inflammatory response (Bainton, Ullyot et al. 1971). IL-1, IL-2, and IL-6 can foster neutrophil development (Heslop, Duncombe et al. 1991; Haylock, To et al. 1992), but many other cytokines, including SCF, G-CSF, and IL-3 are also involved in this process (Cannistra, Koenigsmann et al. 1990; Fibbe, Pruijt et al. 1999).

Both GM-CSF (Lord 1992) and IL-8 (van Eeden and Terashima 2000) can induce dramatically augmented neutrophil mobilization from the marrow sinusoids.

1.2.2.1.3. Neutrophil emigration and activation

Neutrophils have a very short life-span (8-20 hr) in the circulation (Athens, Haab et al. 1961). Once they have migrated into the tissues, they may live 1-4 days (Haslett 1999). Neutrophil transmigration from the vascular compartment into inflamed tissue is important for fighting invading microorganisms and protecting against tissue injury (Smith 1994). This process normally includes neutrophil margination, tethering, rolling, activation, firm adhesion, and transmigration through the vasculature wall, chemotaxis into the tissues, and phagocytosis of foreign microorganisms (Ley 1996).

In the circulation, neutrophils reversibly move from the central blood stream to the periphery of a vessel to form marginating pools, which may contain 55% of the total neutrophils in the circulation (Suwa, Hogg et al. 2000). Marginated neutrophils often reside in the capillaries of specific tissues, such as lung. This special characteristic of the neutrophil facilitates its rapid responses to infection or other stresses (Seely, Pascual et al. 2003). During inflammation, neutrophils start an adhesion cascade comprising capture, rolling, activation and adhesion (Ley 1996). The cascade is initially mediated by P-selectin and E-selectin expressed on vascular endothelial cells, and L-selectin and P-selectin glycoprotein ligand (PSGL)-1 expressed on neutrophils. Expression of these molecules can be stimulated by ELR-CXC chemokines (IL-8, MIP-2), lipid chemoattractants (PAF, LTB_4), cytokines (TNFα, IL-1β), or LPS in the capture and rolling stages. Beta 2-integrins such as LFA-1 and MAC-1 also support rolling. Intercellular adhesion molecule (ICAM)-1 binding to β2-integrins is also important in mediating neutrophil adhesion (reviewed in (Ley 2002; Ley, Laudanna et al. 2007)). Neutrophil activation and arrest requires signaling through ICAM-1, VCAM-1 and integrins, triggered by LPS, IL-8 or other chemoattractants during rolling (Kerfoot and Kubes 2005; Ley, Laudanna et al. 2007). Among these, LPS stimulation of TLR4 on endothelial cells induces rapid P-selectin and E-selectin expression, which results in neutrophil rolling and adhesion (Kerfoot and Kubes 2005). A low concentration of IL-8 is able to stimulate

surface expression and activation of the β2-integrin MAC-1, shedding of L-selectin, and also release of secretory granules. At higher concentrations, IL-8 can induce release of ROS and primary granule mediators such as MPO. C5a can up-regulate β2-integrins and cause shedding of L-selectin on neutrophils (Jagels, Daffern et al. 2000). LTB_4 can also contribute to increased expression of β2-integrin on the neutrophil, which can facilitate neutrophil adherence and migration (Crooks and Stockley 1998). PAF, which is secrected by thrombin-challenged endothelial cells, remains on the surface of endothelial cells and can induce PMN adhesion via its receptor, PAFR (Zimmerman, McIntyre et al. 1990). The signaling component provided by endothelial cell-associated PAF may facilitate activation of the β2-integrins and polarization on PMN and contribute to PMN rolling and firm adhesion (Lorant, Patel et al. 1991; Macconi, Foppolo et al. 1995). PAF may promote TNFα-induced ICAM-1 and E-selectin expression on endothelial cells, which facilitates neutrophil adherence to the endothelium (Sterner-Kock, Braun et al. 1996).

Neutrophil transendothelial migration includes crawling, which is a MAC-1- and ICAM-1-dependent process, and emigration. Emigrating neutrophils need to migrate through three distinct barriers: the endothelium, endothelial basement membrane, and pericytes. Transendothelial migration can be triggered by lumenal chemoattractants such as C5a, PAF, and IL-8 as mentioned above. This process includes both paracellular and transcellular routes. The paracellular route may be associated with increased levels of intracellular endothelial Ca^{2+}, mobilization of platelet/endothelial-cell adhesion molecule (PECAM) -1, -2 and junctional adhesion molecules (JAM) -A, -B, -C. Endothelial cell-selective adhesion molecule (ESAM), as well as the non-immunoglobulin molecule CD99 may be also involved (Ley, Laudanna et al. 2007). The transcellular route is evident when neutrophils migrate into skin stimulated by fMLP (Feng, Nagy et al. 1998), and also occurs in the central nervous system (Engelhardt and Wolburg 2004).

Transmigration through endothelial cells, with stimulation via GPCR ligands such as the ELR-CXC chemokines, C5a, LTB_4, PAF, or fMLP, can lead to full neutrophil activation, rearrangement of their actin cytoskeleton, and migration along a concentration gradient to the sites of inflammation. During this process, they undergo a respiratory burst and degranulation, releasing ROI, and granule components including lactoferrin (LF), myeloperoxidase (MPO), matrix metallopeptidase 9 (MMP-9) and other hydrolytic

enzymes. These inflammatory mediators work together to facilitate killing and digestion of engulfed foreign particles and pathogens or destroying inflamed tissue (Zarbock and Ley 2008).

In the case of pulmonary infections, neutrophils can also migrate through epithelial cell layers to fight invading bacteria or fungi in the airway. Several studies have demonstrated that the ELR-CXC chemokine receptor CXCR2 has a critical role in LPS-induced neutrophil migration into the airway (Reutershan, Morris et al. 2006). C5a and LTB_4 can also facilitate neutrophil transmigration through epithelial cells. In the case of C5a, it can bind its receptor C5aR and stimulate rat alveolar epithelial cells (RAEC) to release TNFα, IL-1β, MIP-2 and CINC-1, which directly facilitate neutrophil transepithelial migration (Carolan, Mower et al. 1997; Riedemann, Guo et al. 2002). LTB_4 contributes to neutrophil transepithelial migration by inducing extracellular signal-regulated kinase (ERK)-mediated ROS signaling in neutrophils (Woo, Yoo et al. 2003). IL-8 and fMLP also induce neutrophil transepithelial migration (Carolan, Mower et al. 1997).

1.2.2.1.4. Neutrophil anti-bacterial mediators

As noted above, once activated, neutrophils respond with an oxidative burst, degranulation of numerous antibacterial proteases, and phagocytosis of particles. Even though these mechanisms play important roles in host defense, they can have the unwanted consequence of damaging normal host tissues as well (Kettritz, Falk et al. 1997). In this section, the origins and the phyisological and pathological effects of neutrophil ROI and granules will be discussed.

Neutrophil ROI release

Reactive oxygen intermediates (ROI) are reactive molecules that can be generated by all mammalian cells. Neutrophil-derived ROI include superoxide (O_2^-), H_2O_2, HO^-, and HOCl (Rosen, Pou et al. 1995). The process of generating these ROI by neutrophils is also termed the respiratory burst due to its associated 50- to 100-fold increase in oxygen

consumption, which occurs after the cells have phagocytosed the invading organisms and incorporated them in their phagosomes. ROI generation begins with the production of superoxide by a plasma membrane-associated, NADPH oxidase complex, during which NADPH oxidase uses NADPH as an electron donor and converts molecular oxygen to its single-electron reduced form, O_2^- (Fialkow, Wang et al. 2007). Superoxide rapidly dismutates to H_2O_2. In the presense of redox-active metal ions, H_2O_2 is converted into hydroxyl radicals (HO^-). When catalyzed by the neutrophil primary granule enzyme MPO in the presense of a halide, H_2O_2 is also converted into hypochlorous acid (HOCl) such as Cl^- (Rosen, Pou et al. 1995; Fialkow, Wang et al. 2007). These ROI are mainly released into phagosomes where they participate in killing bacteria by oxidizing their proteins and lipids. This ROI generation also causes ion flux (e.g., K^+, H^+/Na^+) and pH changes in the phagosome vacuole to promote microbial killing and digestion by optimizing environments for the activation of enzymes. ROI are also released into the cytosol, where they oxidize cellular proteins and lipids thereby modifying their functions (Rosen, Pou et al. 1995; Segal 2006; Fialkow, Wang et al. 2007).

Although ROI are believed to be involved in microbial killing simply by reacting with organic molecules, they actually contribute on several different levels. Superoxide does not kill bacteria directly. H_2O_2 has a bactericidal effect at high concentrations (e.g., 100mM), but HOCl is the most lethal of the ROI to bacteria (Reeves, Nagl et al. 2003). Due to its short half-life and ability to diffuse only short distances, HO^- may primarily act on alternate targets before actually reaching the bacterium, and therefore have little impact on the killing process (Hampton, Kettle et al. 1998). But some studies question if these ROI have direct bactericidal effects in the phagosome. They found that there is both a high concentration of granule proteins (e.g., 500 mg/ml) and a high pH (pH ~7.8) environment in the phagosome at the time of bacterial death, which may completely inhibit the bactericidal effects of ROI. Some also think that MPO may play an important role in protecting the microbicidal enzymes against oxidative damage by ROI (Reeves, Nagl et al. 2003; Segal 2005). Other studies do not agree with this point and suggest that ROS and MPO are essential in killing some strains of bacteria. The mechanism of protease release from neutrophils into the extracellular environment remains to be explored (Roos and Winterbourn 2002).

High concentrations of ROI can be released into the extracellular environment and cause tissue injury. ROI can directly activate the transcription factor NF-κB and thereby indirectly regulate proinflammatory cytokine and chemokine expression by neutrophils. ROI can induce phosporylation of focal adhesion kinase in endothelial cells, which leads to dysfunction of the pulmonary endothelial barrier and increases permeability. ROI also participate in the regulation of neutrophil apoptosis through their impact on protein tyrosine kinases, protein tyrosine phosphatases, and inositol phosphatases (Kettritz, Falk et al. 1997; Moraes, Zurawska et al. 2006; Fialkow, Wang et al. 2007). This process can be important for the removal of neutrophils that have lost function, but it can also damage other normal residential cells.

ROI are essential for killing invading pathogens, but also damage normal host cells causing tissue injury, such that their regulation is extremely important. Neutrophils have evolved pathways to restrict ROI-induced pathology as well, including superoxide dismutases for superoxide and glutathione peroxidase and catalase for hydrogen peroxide (Rosen, Pou et al. 1995; Fialkow, Wang et al. 2007).

Neutrophil degranulation

Another important neutrophil microbicidal mechanism is degranulation. When neutrophils bind opsonized microorganisms and phagocytosis is triggered, neutrophils quickly release dramatic amounts of granule proteins (as high as 500 mg/ml) including MPO and elastase (primary granules), lactoferrin (secondary granules), and MMP-9 (tertiary granules), into the phagosome to digest target proteins (Smith 1994; Segal 2005).

MPO and Elastase

Primary granules are also traditionally referred to as “azurophil granules” due to their affinity for the basic dye azure A. Myeloperoxidase, elastase, and defensins are important components of this group of granules. MPO is a 150-kDa microbicidal hemoprotein which can be released into the phagosome or to the extracellular space upon neutrophil activation. MPO reaction with H_2O_2, as mentioned above, is an important

mechanism to produce other more potent microbicidal ROI, such as HOCl and chlorination products. These ROI can attack the surface membranes of microorganisms and, when released to the outside of the cells, cause damage to tissues, such as the epithelium (Faurschou and Borregaard 2003; Klebanoff 2005). As previously mentioned, some reports suggest that MPO only functions as a catalase and protects digestive enzymes from oxidative denaturation. But it has a microbicidal capacity in the extracellular environment where enzyme concentrations and the pH are low and it conducts halogenation reactions (Segal 2005). Elastase, a serine proteinase, is also a component of the primary granule. It plays an important role in killing bacterium in the phagosome. Neutrophil elastase-deficient mice were found to be excessively susceptible to infection by Gram-negative bacteria (e.g., *E. coli*), rather than Gram-positive bacteria (e.g., *S. aureus*). But if released into the extracellular milieu, it can also damage host cells such as epithelial cells. Elastase can induce apoptosis of pulmonary epithelial cells *in vitro*, which indicates that elastase may contribute to disrupted epithelial cell barrier function and cause alveolar edema (Moraes, Zurawska et al. 2006). Elastase may also be involved in upregulating IL-8 production by the bronchial epithelium (Devaney, Greene et al. 2003).

Lactoferrin

One representative neutrophil specific granule component is LF, a 78-kDa glycoprotein. It is also a member of a transferring family of ion-binding proteins. Lactoferrin functions as a bactericidal effector through sequestration of iron from bacteria, leading to impaired bacterial growth and finally, death. Due to its ability to sequestrate environmental iron, it is involved in regulating H_2O_2 production. It also can bind to bacterial cell membranes via an N-terminal amphipatic α-helical region, leading to cell membrane damage and lysis (Rosen, Pou et al. 1995; Faurschou and Borregaard 2003). In addition, LF acts as a regulator for neutrophil aggregation and their adherence to endothelial cells thereby amplifying the inflammatory response (Oseas, Yang et al. 1981).

MMP-9 and MMP-2

Neutrophil tertiary granules contain metalloproteases such as MMP-9. MMP-9 is a 92 kDa protein and is also called gelatinase B, due to its ability to digest the extracellular matrix structure, gelatin (Gelatinase A is MMP-2, another kind of metalloprotease) (Opdenakker, Van den Steen et al. 2001; Faurschou and Borregaard 2003). MMP-9 is mainly produced by inflammatory cells such as neutrophils and monocytes/macrophages. In neutrophils, MMP-9 is stored as an inactive proform in granules and is released during excyotosis, while macrophages synthesize MMP-9 *de novo* upon stimulation (Warner, Bhagavathula et al. 2004). It is also released by resident cells such as pulmonary fibroblasts and type II bronchial epithelial cells, but not pulmonary artery endothelial cells (Warner, Bhagavathula et al. 2004). The stimuli can be bacterial (e.g., LPS) or viral products (e.g., double-stranded RNA), cytokines (e.g., TNF, IL-1β) or chemokines (e.g., IL-8) (Opdenakker, Van den Steen et al. 2001). MMP-2 is mainly produced by resident cells such as fibroblasts and epithelial cells (Warner, Bhagavathula et al. 2004). MMP-9 and MMP-2 play major roles in degrading vascular basement membranes and interstitial structures during neutrophil transendothelial and transepithelial cell migration. Therefore, for neutrophils, they are the main granules that increase vascular permeability and pulmonary edema during neutrophilic inflammation and are often used as surrogate measures for inflammatory responses (Corbel, Boichot et al. 2000; Eichler, Bechtel et al. 2003).

MMP-9 has other functional roles. It facilitates the activation of pro-IL-1β into active IL-1β. It can also truncate the amino terminus of IL-8 and convert IL-$8_{(1\text{-}77)}$ into IL-$8_{(7\text{-}77)}$. IL-$8_{(7\text{-}77)}$ possesses dramatically higher CXCR1/2 binding affinity and increased abilities to induce the intracellular calcium flux, chemotactic responses, and MMP-9 release in neutrophils. Thus, MMP-9 is not only an effector but also a regulator of neutrophil functions (Opdenakker, Van den Steen et al. 2001). To prevent tissue injury by MMPs, tissue inhibitors of MMPs (TIMPs) are also generated by cells such as monocytes. Neutrophils do not produce TIMP-1 or the MMP/TIMP-1 complex (Opdenakker, Van den Steen et al. 2001).

1.2.2.1.5. Neutrophil apoptosis

Neutrophils have a specified short life-span in the circulation and tissues as mentioned previously. A prolonged neutrophil life-span may be beneficial in the context of anti-microbial activities (Kobayashi, Voyich et al. 2005), but it also brings the risk of causing tissue damage, especially if excessive or inappropriate activation occurs. For that reason, neutrophil homeostasis and turnover are elaborately regulated (Luo and Loison 2008).

Programmed cell death is the main mechanism that regulates neutrophil homeostasis. During programmed cell death neutrophils die through apoptosis or other pathways, although apoptosis is the most important of these. For neutrophils, the timing of apoptosis during the course of development is genetically determined (Tang, Mura et al. 2008). Generally, apoptosis is characterized by cell shringkage, DNA cleavage, nuclear fragmentation, and nuclear dissolution, although the plasma membrane remains intact and retains cytosolic enzymes and other pro-inflammtory agents. However, cells lose their functions such as chemotaxis, respiratory burst, intracellular calcium flux and degranulation under the influence of external stimulation. The cell surface receptors and adhesion molecules are also downregulated. In the later stages, the cells break up into apoptotic bodies and are finally phagocytosed by macrophages, and other neutrophils (Haslett 1997; Seely, Pascual et al. 2003; Serhan and Savill 2005; Luo and Loison 2008).

In addition to such genetically-controlled "programmed" death, neutrophils can be triggered by external stimuli such as soluble cell death ligands (e.g., TNF or Fas ligand) which bind TNF receptor I or Fas, respectively. Neutrophil apoptosis can also be triggered by intrinsic stimuli such as ionizing radiation, or cytokine deprivation (Ashkenazi and Dixit 1998; Kroemer and Martin 2005). Apoptosis is characterized by caspase activation and the lack of inflammation, which is distinct from other forms of cell death such as oncosis, autophagy, and capase-independent death (Simon 2003; Tang, Mura et al. 2008). Alteration of cell membrane expression is an important feature of apoptotic neutrophils, such that phosphatidylserine, which normally resides on the inner membrane leaflet, is expressed instead on the outer membrane at the early stage of neutrophil apoptosis. Therefore, macrophages can easily recognize apoptotic cells (Fadok, Savill et al. 1992).

Phosphatidylserine expression on the outer membrane can be specifically detected by fluoresecent-labeled ligands (e.g., annexin V) as a means of detecting apoptotic cells (Vermes, Haanen et al. 1995).

During inflammation, a number of inflammatory mediators have an anti-apoptotic effect on neutrophils, including ELR-CXC chemokines (IL-8, GROα) (Kettritz, Gaido et al. 1998; Klein, Rane et al. 2000; Glynn, Henney et al. 2002), bacterial peptide (fMLP), C5a, LPS, and GM-CSF (Lee, Whyte et al. 1993; Kobayashi, Voyich et al. 2005). Both IL-8 and GROα are able to delay neutrophil apoptosis, but IL-8 is more effective than GROα. Noteably, IL-8 is also able to inhibit TNFα-mediated neutrophil apoptosis. The neutrophil anti-apoptotic processes induced by IL-8 are mediated by the extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways (Kettritz, Gaido et al. 1998; Klein, Rane et al. 2000). C5a is also able to dose-dependently delay neutrophil apoptosis, and this is also related to PI3K pathway activation (Perianayagam, Balakrishnan et al. 2002). Delayed neutrophil apoptosis can also be induced by LPS through binding TLR4 on neutrophils, and activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways (i.e. not PI3K-independent pathway). LPS-TLR4 binding plays a significant role in inducing anti-apoptotic effects in neutrophils although prolonged neutrophil survival requires both LPS-induced TLR4 activation and monocytes (leading to expression of additional survival factors; G-CSF) (Yamamoto, Yoshida et al. 1993; Sabroe, Prince et al. 2003). GM-CSF and G-CSF can also delay neutrophil apoptosis (Klein, Rane et al. 2000; van Raam, Drewniak et al. 2008), and in doing so contribute to the severity of pulmonary neutrophilia in ARDS patients, although G-CSF and IL-8 play more important roles than GM-CSF (Aggarwal, Baker et al. 2000).

As activated neutrophils play a major role in the pathogenesis of many diseases (e.g., ARDS, sepsis), the resolution of neutrophil-induced inflammatory responses relies on reversing delayed neutrophil apoptosis. Studies using the ELR-CXC chemokine receptor CXCR2 antagonist SB272844 demonstrated that blocking CXCR2 can effectively reverse IL-8- and GROα-induced anti-apoptotic effects in neutrophils (Glynn, Henney et al. 2002). This study indicated that strategies can be used to reverse the anti-apoptotic effects of ELR-CXC chemokines by blocking their receptors.

1.2.2.1.6. Roles of neutrophils in inflammatory diseases

It is well established that excessive neutrophil recruitment and activation can cause tissue injury and even host death (Lee and Downey 2001). So far, neutrophils have been found to play a pivotal role in mediating the pathology of a number of inflammatory diseases, including ALI/ARDS (Lee and Downey 2001), COPD (Tetley 2005), I/R injury (Belperio, Keane et al. 2005), IBD (Ina, Kusugami et al. 1997), and RA (Grespan, Fukada et al. 2008). Neutrophils function as important effector cells of tissue pathology by releasing cytotoxic mediators such as ROI, cationic peptides, eicosanoids, and proteolytic enzymes which can damage normal tissue and cause tissue injury (Lee and Downey 2001). ROI, including superoxide (O_2^-), H_2O_2, and HOCl, together with proteolytic enzymes such as MPO, elastase, and MMP-9, are responsible for oxidative tissue injury and digestion of intercellular and extracellular matrices. The damage increases vascular permeability and causes endothelial and epithelial cell damage, leading to organ dysfunction. For instance, MPO facilitation of HOCl and elastase production contributes to emphysema in COPD patients (Tetley 2005). Elastase can also up-regulate IL-8 expression by epithelial cells in cystic fibrosis (Devaney, Greene et al. 2003), and MMP-9 is related to alveolar-capillary permeability increases in ARDS patients (Delclaux, d'Ortho et al. 1997). Moreover, having activated circulating neutrophils is a prerequisite to multiple organ dysfunction syndromes (MODS) in ischemia and reperfusion injury (Carden and Granger 2000). Activated neutrophils can release cytokines (TNF, IL-1, and IL-6) (Nikolaus, Bauditz et al. 1998), chemokines (IL-8) and other chemoattractants (e.g., LTB4) that amplify the pathology, as occurs in RA (Chen, Lam et al. 2006). Neutrophil release of IL-8 and LTB4 may also correlate with the level of neutrophil inflammation and the degree of airflow limitation in the lungs of COPD patients (Tetley 2005).

1.2.2.2. Monocytes/macrophages

Monocytes originate in the bone marrow from a common myeloid progenitor. After they are released into the blood, they circulate for several days before entering tissues and differentiating into a variety of tissue macrophage populations such as alveolar macrophages (AMs), or more specialized cells such as dendritic cells (DCs) (Gordon and

Taylor 2005). Alveolar macrophages can be derived both directly from precusors in the peripheral blood and from proliferation of local resident precursors. In the short term, AMs arise mostly from local proliferation, but over a longer period, they can be replenished from the bone marrow (Kennedy and Abkowitz 1997). In the physiological state, AMs are long-lived cells that turn-over slowly, with a replacement rate of ~40% per year (Kennedy and Abkowitz 1997; Maus, Janzen et al. 2006), but during acute lung inflammation (e.g., airway endotoxemia) 61% of these cells are replaced within two months (Maus, Janzen et al. 2006). Further studies have demonstrated that blood monocytes are a heterogenous population. Studies in mice have identified two functional subsets of blood monocytes, including $CX3CR1^{lo}CCR2^{+}Gr1^{+}$ (also referred to as $CD14^{+}$) and $CX3CR1^{hi}CCR2^{-}Gr1^{-}$ (also referred to as $CD16^{+}$) (Geissmann, Jung et al. 2003; Serbina, Jia et al. 2008). $CX3CR1^{lo}CCR2^{+}Gr1^{+}$ monocytes are short-lived inflammatory monocytes and are actively recruited to inflammated tissues to differentiate into macrophages or antigen-presenting DCs. $CX3CR1^{hi}CCR2^{-}Gr1^{-}$ monocytes persist longer in tissues and serve as precursors for resident macrophages (Geissmann, Jung et al. 2003; Landsman, Varol et al. 2007). There is direct evidence that alveolar macrophages arise from blood monocytes, but via lung macrophage intermediaries (Landsman and Jung 2007). These studies suggested that blood monocytes are able to differentiate into normal tissue resident macrophages over a long term (e.g., 2 -12 mo), but differentiate into "inflammatory monocytes" rapidly (e.g., in 1 day).

Blood monocytes already have phagocytic capabilities and express receptors for IgG Fc-domains (FcγR) and C3b. After migrating into tissues, they undergo further differentiation to become multifunctional tissue macrophages. Compared with tissue macrophages, monocytes retain greater proliferative capacity, but a decreased ability to phagocytose as well as fewer lysosomes and IgG receptors (Cline, Lehrer et al. 1978). They also express fewer TLRs and scavenger receptors (Gordon and Taylor 2005). In addition to phagocytosis, alveolar macrophages have two other important functions, presentation of antigen to T-cells and immunomodulation. Therefore, AMs function as the first line of cellular defense against respiratory pathogens. In the functional role of phagocytosis, AMs phagocytose two kinds of material: waste and debris (e.g., dead cells) and invading pathogens. As discussed above, the phagocytosis of apoptotic neutrophils by

AMs plays a crucial role in maintaining the homeostasis of neutrophils and in the resolution of inflammation (Fadok, Savill et al. 1992; Haslett 1999). Alveolar macrophages also phagocytose foreign pathogens by recognizing microbes through their cell surface receptors such as, TLRs (e.g., TLR2, TLR4), scavenger receptors (SR, e.g., SR-A), and Fc and C' receptors (Palecanda, Paulauskis et al. 1999; Fujiwara and Kobayashi 2005). They also produce large amounts of oxygen radicals and proteolytic enzymes to destroy engulfed foreign microorganisms. Another role of AMs is to present antigens for recognition by T cells and to further activate lymphocytes, which in turn activate macrophages to kill the microbes.

In addition to these two important functions, AMs can also release various proinflammatory cytokines (e.g., TNFα, IL-1β, IL-6) (Kotloff, Little et al. 1990; Ulich, Watson et al. 1991; Beck-Schimmer, Schwendener et al. 2005), chemokines (IL-8, MIP-2) (Kooguchi, Hashimoto et al. 1998; Palmberg, Larsson et al. 1998), and other chemoattractants (e.g., LTB_4) (Christman, Petras et al. 1989) upon contact with foreign pathogens or their products (e.g., LPS, bacteria). Through these inflammatory mediators, AMs play a key role in initiating inflammatory responses and regulating pulmonary neutrophil migration and activation, and therefore exacerbation of lung pathology. Among them, the chemokines (e.g., MIP-2, KC) play a major role in mediting neutrophil recruitment (Gupta, Feng et al. 1996). On the other hand, a study found that AMs also play a regulatory role in inflammatory responses. As noted above, AMs are able to engulf apoptotic neutrophils, but at the same time they release anti-inflammatory mediators (e.g., transforming growth factor β1; TGF β1) which suppress inflammatory responses. At the same time, the production of proinflammtory cytokines is also inhibited through autocrine/paracrine mechanisms (Fadok, Bratton et al. 1998; Huynh, Fadok et al. 2002). Monocyte recruitment to the airway in acute inflammatory conditions (e.g., 24 h after monocyte chemotactic protein-1 [MCP-1] adminsitration) may amplify inflammatory responses through increased expression of TNFα, neutrophil chemokines (e.g., MIP-2, KC), and extracellular matrix degradation (Srivastava, Jung et al. 2005). As mentioned earlier, monocyte recruitment by MCP-1 facilitates neutrophil migration and activation after intratracheal LPS challenge (Maus, Waelsch et al. 2003). Neutrophil migration may also contribute to monocyte recruitment. Studies have found depletion of neutrophils before

intratracheal administration of LPS abrogated the early (3 h) as well as late (24 h) monocyte/macrophage increases in the lung (Janardhan, Sandhu et al. 2006).

As discussed above, monocytes and macrophages play crucial roles in phagocytosis of foreign microorganisms, regulation of neutrophil recruitment and activation, and immunomodulation of inflammation. Compared with neutrophils, monocytes and macrophages have some distinct features. First of all, they stay in the circulation or tissues longer and they can proliferate. Second, they, especially macrophages, can kill a more diverse array of microbes. Third, there are fewer monocytes in the blood (e.g., 10%, versus 60-70% for neutrophils) and at the site of severe inflammation, macrophages need to recruit neutrophils to function as the primary phagocytes. Fourth, they accumulate more slowly and have a less extreme metabolic burst. Finally, macrophages not only initiate, but also resolve, inflammation. Neutrophils may be thought as the soldiers in an army, but macrophages have leadership roles. Generally, neutrophils play a more important role in fighting foreign antigens and, if inappropriately activated, also mediating excessive inflammatory responses (Dale, Boxer et al. 2008).

1.2.2.3. Eosinophils

Eosinophils are important effector cells in allergic diseases such as asthma and parasitic infections (Yang, Loy et al. 1998). Studies have indicated that C-C chemokines are important for eosinophil recruitment and activation during allergic airway responses. These include eotaxin, RANTES (regulated upon activation, normal T cell expressed and secreted) and MIP-1α (macrophage inflammatory protein -1α) (Lukacs, Standiford et al. 1996; Yang, Loy et al. 1998). Eosinophils are considered to be important effector cells for killing certain parasitic pathogens, but can also cause direct damage to normal host tissues through a variety of mechanisms. Activated eosinophils can release toxic products such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and oxygen radicals. These molecules are toxic to the airway epithelium and can damage bronchial structures, thereby increasing bronchial hyperresponsiveness, resulting in lung dysfunction (Kroegel, Warner et al. 1994; Oddera, Silvestri et al. 1998). In addition, eosinophils produce a variety of cytokines such as IL-4, -5, and -6 and interferon-

γ (IFN-γ) and play important roles in regulating the proliferation and differentiation of T and B lymphocytes (Kroegel, Warner et al. 1994).

1.2.2.4. Mast cells

Mast cells are another cell type that is important in allergic inflammatory diseases. Mast cells can be activated by cross-linking the $Fc_{\varepsilon}RI$-receptor after binding of IgE, recognizing specific antigens. They can also be activated by I/R-induced ROI and complement split products (Schramm and Thorlacius 2004). The activation of mast cells results in the release of multiple cytokines including IL-4,-5,-6, and TNFα, and chemokines such as IL-8. They also express CXCR1 and CXCR2 on their cell surface and can be chemoattracted by IL-8 binding CXCR2 (Lippert, Artuc et al. 1998; Nilsson, Mikovits et al. 1999). Importantly, mast cells have been shown to contribute to recruitment of neutrophils through their released cytokines (e.g., TNFα) and chemokines (e.g., MCP-1) (Maus, Waelsch et al. 2003; Schramm and Thorlacius 2004).

1.2.2.5. Endothelial cells

Endothelial cells play a variety of roles in inflammation. These include wound healing, angiogenesis, and leukocyte trafficking (Swerlick and Lawley 1993). In acute lung injury, endothelial cells contribute to initiating inflammation, facilitating neutrophil migration, and increasing vascular permeability through the production of cytokines (e.g., TNFα, IL-1), chemokines (e.g., IL-8), adhesion molecules (e.g., VCAM-1), and vasodilator factors (e.g., prostacyclin; PGI_2) (Mantovani, Bussolino et al. 1992).

First of all, endothelial cells play an important role in initiating inflammation. In ischemia/reperfusion injury, hypoxia-reoxygenation induces endothelial cells to generate ROI, resulting in production of proinflammtory mediatiors (e.g., TNFα, IL-1, PAF). These molecules directly or indirectly amplify the inflammatory responses (Grace 1994). Second, as mentioned above, endothelial cells generate a variety of inflammatory mediators, such as cytokines. Studies have demonstrated that endothelial cells can produce TNFα and IL-1 when challenged by LPS and TNFα, respectively (Nawroth, Bank et al. 1986; Ranta,

Orpana et al. 1999). These two cytokines can activate yet more endothelial and epithelial cells (Smart and Casale 1994; McHale, Harari et al. 1999) to produce chemokines (e.g., IL-8). Third, they significantly contribute to neutrophil migration by expressing adhesion molecules and releasing chemoattractants. Endothelial cells express adhesion molecules such as ICAM-1 and VCAM-1 when stimulated by TNFα and IL-1 (McHale, Harari et al. 1999), PAF and LTB_4 under hypoxia-reoxygenation challenged conditions (Grace 1994), and chemokines such as IL-8 (Smart and Casale 1994). These molecules are important in the facilitation of neutrophil activation and transendothelial migration.

Human umbilical vein endothelial cells (HUVEC) express CXCR1 and CXCR2 (Kalia, Brown et al. 2003; Li, Varney et al. 2005), while pulmonary endothelial cells reportedly express only the CXCR2 (Reutershan, Morris et al. 2006). In a murine model of LPS-induced lung injury, endothelial cell CXCR2 was a crucial factor involved in neutrophil recruitment and pulmonary vascular leakage (Reutershan, Morris et al. 2006). Endothelial cell-derived IL-8 generates the required chemotactic gradients in the subendothelial matrix to regulate neutrophil transmigration (Detmers, Lo et al. 1990; Huber, Kunkel et al. 1991). Endothelial cells also express C5a, LTB_4, and PAF receptors (i.e. C5aR, BLT, and PAF), and their ligands can stimulate endothelial cells to produce adhesion molecules, cytokine, and chemokines, which facilitate neutrophil migration. For instance, C5a can interact with endothelial cells to stimulate P-selectin expression (Ward 1996). LTB_4 does not stimulate endothelial cells to produce IL-8 or PAF (McIntyre, Zimmerman et al. 1986; Nohgawa, Sasada et al. 1997), but *in vitro* experiments indicate that HUVEC express LTB_4 receptors (BLT_1 and BLT_2) when stimulated by LPS and TNFα and show higher responsiveness to LTB_4. LTB_4 can augment nitric oxide and MCP-1 production from HUVEC stimulated by LPS (Qiu, Johansson et al. 2006). PAF may promote TNFα-induced ICAM-1 and E-selectin expression on endothelial cells, which facilitates neutrophil adherence to the endothelium (Sterner-Kock, Braun et al. 1996). As discussed above (§ 1.2.1.4 and § 1.2.2.1.3), endothelial cell TLR4 signaling by LPS play a major role in facilitating neutrophil migration in systemic endotoxemia, but play a less important role in airway endotoxemia when compared with LPS-TLR4 signaling on macrophages (Hollingsworth, Chen et al. 2005).

Activation of endothelial cells with TNFα and IL-1 leads to increased vascular permeability through release of vasodilator factors such as PGI_2 (Mantovani, Bussolino et al. 1992). Neutrophil and endothelium interaction (via ICAM-1) is also an important factor that affects pulmonary vascular permeability (Sumagin, Lomakina et al. 2008).

In general, endothelial cells contribute to inflammatory responses and tissue pathology in several ways. Among these, the key roles are facilitating neutrophil activation and migration and changing vascular permeability, in which IL-8 and neutrophil-endothelial interactions are extremely important.

1.2.2.6. Epithelial cells

Epithelial cells make up the epithelia that line the cavities and surfaces of structures throughout the body, such as the lung and gastrointestinal tract. In different tissues, epithelial cells may play different roles. Those lining the airways, as a primary interface between pathogens and the environment, play a crucial role in the innate immune response (Becker, Diamond et al. 2000). Ciliated epithelial cells in the upper airway play an important role in clearing materials with ciliary movement and form a biological barrier to protect against foreign pathogens (Cantin 2001). The alveolar epithelium consists of alveolar type I and type II cells, which have distinct functions. Type I cells comprise 90% of the alveolar surface and are important for the exchange of respiratory gases. Type II alveolar cells are multifunctional cells. They can produce surfactant and play an important role in alveolar liquid and bacterial clearance (Cantin 2001; Geiser 2003). They also release cytokines and chemokines, the discussion of which will follow.

Airway epithelial cells can be activated by AM-originated cytokines (e.g., TNFα IL-1β), complement (e.g., C5a), and bacterial products (e.g., LPS) to produce cytokines (TNFα, IL-1β, and IL-6), and chemokines (e.g., IL-8, MIP-2, CINC) (Liu, Mul et al. 1996; Allen, Menendez et al. 2000; Becker, Diamond et al. 2000; Riedemann, Guo et al. 2002). The human tracheobronchial epithelium also releases β defensin-2, a key antibacterial molecule, after challenge with LPS (Becker, Diamond et al. 2000). Airway epithelial cells also express TLRs, such that when challenged by TLR ligands such as LPS or double stranded RNA (dsRNA), they can release IL-8 and IL-6. Compared with other TLR

ligands, dsRNA induces more IL-8 production (Sha, Truong-Tran et al. 2004). When exposed to foreign particulate matter, AMs and epithelial cells diplay a significant synergy in production of IL-1β, IL-6, IL-8 and GM-CSF (Fujii, Hayashi et al. 2002). From these studies, it is easy to conclude that airway epithelial cells play a critical role in the production of cytokines and chemokines, particularly IL-8. These mediators can amplify the inflammatory responses of epithelial cells and other resident cells (e.g., macrophages, endothelial cells) via further cytokine production and neutrophil activation and migration.

Similar to the endothelium, the epithelium is also actively involved during neutrophil infiltration into the sites of inflammation (e.g., airway), although epithelial cells express fewer types of adhesion molecules. The adhesion molecule ICAM-1 is expressed on epithelial cells. Its ligand on the neutrophil, CD11b/CD18, is critical to neutrophil transepithelial migration, but the role of ICAM-1 in mediating neutrophil migration still needs be further studied (Mul, Zuurbier et al. 2000; Chin and Parkos 2007).

During neutrophil transepithelial migration, chemoattractants play important roles. fMLP is a potent chemotactic factor, as are LTB4, C5a, and IL-8 (Chin and Parkos 2006). fMLP has greater potency than LTB_4 on chemoattracting neutrophils migration through the epithelium (Casale, Abbas et al. 1992). LTB_4 facilitates neutrophil transepithelial cell migration through a ROS-mediated ERK linked cascade (Woo, Yoo et al. 2003). Pulmonary epithelial cells express C5aR, such that C5a can activate rat alveolar epithelial cells. It also has a synergistic effect with LPS and TNFα in the stimulation of epithelial cells to release cytokines (Riedemann, Guo et al. 2002), which facilitates neutrophil recruitment. As previously discussed, IL-8 is an extremely important product of and stimulus for epithelial cells. Pulmonary epithelial cells reportedly express the neutrophil receptor CXCR2 (Reutershan, Morris et al. 2006), while intestinal tissue epithelial cells express only the CXCR1 (Sturm, Baumgart et al. 2005). Pulmonary epithelial cells that express CXCR2 are believed to be involved in neutrophil recruitment and lung permeability, which would implicate the ELR-CXC chemokines in this process. IL-8 production by both airway and intestinal epithelial cells can feed into an autocrine mechanism (Patel, Jiang et al. 1998; Maheshwari, Lacson et al. 2004), which is an important role for IL-8 in epithelium injury. In the case of colonic epithelial cells, IL-8 has an important role in stimulating epithelial cell migration and helping to maintain the

mucosal epithelial surface barrier, which may contribute to protecting the host against the penetration of inflammatory agents (Sturm, Baumgart et al. 2005).

Epithelium damage significantly affects lung function, causing protein leakage into the airway. Neutrophil transepithelial migration plays an important role in mediating epithelial permeability. Proteinases released by neutrophils (e.g., elastase) during transepithelial migration contribute to intestinal epithelial permeability. It causes epithelial loss and the disruption of the epithelial barrier (Ginzberg, Cherapanov et al. 2001). In the airway, neutrophil-derived elastase, acting via proteinase activated receptor-1, can induce apoptosis of lung epithelial cells, which thereby can increase the permeability of the alveolar capillary barrier, resulting in blood protein leakage (Suzuki, Moraes et al. 2005). Another study with using a neutrophil elastase inhibitor (ONO-5046) also supports that elastase is involved in increasing alveolar epithelial permeability (Miyazaki, Inoue et al. 1998). The tight adherence of stimulated neutrophils to epithelial cells and the production of serine proteases appear to promote epithelial cell killing (Simon, DeHart et al. 1986; Chin and Parkos 2007).

1.2.3. ELR-CXC chemokine biology

1.2.3.1. Human, rat and guinea pig ELR-CXC chemokines and their receptors

The Glu-Leu-Arg (ELR)-CXC chemokines, a subfamily of the CXC chemokines, are known to be primary agonists for neutrophil recruitment. In humans, ELR-CXC chemokines include CXC ligand 1 (CXCL1) (growth-related oncogene; GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL5 (epithelial neutrophil-activating peptide-78; ENA-78), CXCL6 (granulocyte chemotactic protein-2; GCP-2), CXCL7 (neutrophil-activating peptide-2; NAP-2), and CXCL8 (IL-8). Rats possess CINC-1 (GRO/KC), -2α, -2β, and -3 (MIP-2) (Dunstan, Salafranca et al. 1996; Shibata, Konishi et al. 2000). In guinea pigs, CXCL8 and GRO are important members and share 30-45% amino acid homology with their human homologues (Yoshimura, Takeya et al. 1999). Human and guinea pig ELR-CXC chemokines chemoattract and activate neutrophils via their CXCL8 receptors, CXCR1 and CXCR2 (Murphy and Tiffany 1991; Ahuja and Murphy 1996; Catusse, Faye et al. 2003; Takahashi, Jeevan et al. 2007) and also exhibit potent angiogenic activity. Rat

ELR-CXC chemokines bind to target cells only via the CXCR2, even though homologues of both CXCR1 and CXCR2 have been discovered (Dunstan, Salafranca et al. 1996; Shibata, Konishi et al. 2000). Human CXCR1 is specific for CXCL8, to which it binds with high affinity, but it also binds GCP-2 and NAP-2 with lower affinity (Schnitzel, Garbeis et al. 1991; Ahuja and Murphy 1996). CXCR2 similarly binds CXCL8 with high affinity, but also binds MIP-2, CINC-1 (rodents), GROα, -β, -γ, ENA-78, GCP-2, and NAP-2 with somewhat lower affinities (Schnitzel, Garbeis et al. 1991; Loetscher, Seitz et al. 1994; Ahuja and Murphy 1996; Richardson, Pridgen et al. 1998; Wuyts, Proost et al. 1998; McColl and Clark Lewis 1999).

1.2.3.2. The structure of ELR-CXC chemokines and their receptors

As a subgroup of the CXC chemokine, the ELR-CXC chemokines contain four conserved cysteines and there is one alternate amino acid between first two cysteines (e.g., C<u>X</u>C). In addition, the ELR-CXC chemokines have a special tripeptide motif, Glu-Leu-Arg (ELR) motif in the amino-subterminal region, immediately distal to the first cysteine. These four cysteines form two disulphide bonds (Cys^{7}-Cys^{34} and Cys^{9}-Cys^{50}) which confer three-dimensional folding that is essential for receptor recognition and biological activity (Baggiolini 2001).

1.2.3.3. Molecular biology of CXCL8

Among the ELR-CXC chemokines, CXCL8's structure and function are well studied. The human CXCL8 cDNA encodes a 99-amino acid precursor protein with a signal sequence, which is cleaved to yield 77- or 72-residue mature proteins, of which the functional protein is 72 amino acids (Matsushima, Morishita et al. 1988). CXCL8 exists as a monomer and dimer at nanomolar concentrations. Its dimerization constants range from about 100nM to 100μM *in vitro*. But basically, the monomer is the functional form of CXCL-8 (Rajarathnam, Sykes et al. 1994). The monomeric unit contains a highly flexible NH_2-terminal region followed by a series of loops and turns (also called an N-loop), three antiparallel β strands and an overlying COOH-terminal α-helix. Among them, the NH_2-

terminal Glu^4-Leu^5-Arg^6 (ELR) motif and Ile^{10} are essential for receptor binding and signaling (Clark-Lewis, Dewald et al. 1994; Rajagopalan and Rajarathnam 2004). The N-loop formed by Tyr^{13}, Ser^{14} Lys^{15}, Phe^{17}, His^{18} and Phe^{21} and the surface hydrophobic pocket formed by residues Tyr^{13}, Ser^{14}, Phe^{21}, and Lys^{49} and/or adjacent residues including Leu^{25} and Leu^{43}, participate in IL-8 receptor-selective recognition for both the CXCR1 and CXCR2. It is thought that the hydrophobic pocket itself may be essential for CXCR1 (Schraufstatter, Ma et al. 1995; Hammond, Shyamala et al. 1996; Wells, Power et al. 1996; Suetomi, Lu et al. 1999). Residues 30-35, especially Gly^{31} and Pro^{32}, have important structural roles. The Cys^7-Cys^{34} disulfide bridge anchors the ELR region to the '30-35' turn which provides the correct geometry for the Cys^7-Cys^{34} disulfide bridge and determines the conformation of the ELR motif (Clark-Lewis, Dewald et al. 1994) (see Figure 1.1.). The importance of Gly^{31} and Pro^{32} suggests that changing them may affect the framework for the receptor binding motif and receptor signaling as well. This topic will be further discussed below.

The ELR-CXC chemokines are expressed by many cell types in response to inflammatory stimuli (Gerard and B 2001). CXCL8, for example, can be produced by leukocyte cells (monocytes, T cells, neutrophils, and natural killer cells) and non-leukocytic somatic cells (endothelial cells, fibroblasts, and epithelial cells) (Mukaida 2000; Mukaida 2003). CXCL8, the prototypical ELR-CXC chemokine, is regulated by nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), each of which bind the distal (3') end of the CXCL8 promoter as dimers, either individually or in collusion with one another (Roebuck 1999). Environmental factors can induce CXCL8 production in several types of cells. Hypoxic conditions induce tumor cells to produce high levels of CXCL8 through the cooperative activation of AP-1 and NF-κB (Xu, Xie et al. 1999). Because CXCL8 has potent angiogenic activities (Strieter, Polverini et al. 1995), hypoxic conditions may induce neovascularization by inducing the production of CXCL8. Reactive oxygen intermediates can activate NF-κB, an essential transcription factor for CXCL8 gene, and thereby induce CXCL8 gene transcription (Lee, Shin et al. 2008). These findings suggest that ELR-CXC chemokines may play an important role in cancer and ischemia/reperfusion injury (e.g., both characterized by hypoxia of the ischemic organ and large amounts of ROI production).

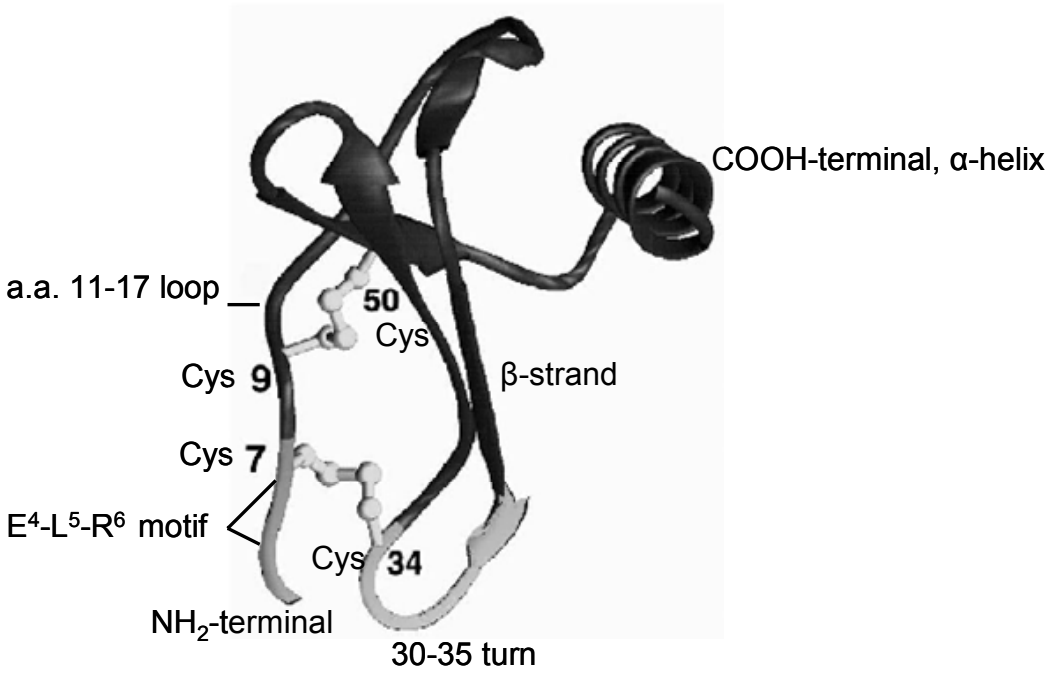


Figure 1.1. Ribbon diagram of the human IL-8 molecule.

The structure of IL-8 contains a flexible NH2-terminal section including a receptor signaling motif (E^4-L^5-R^6) followed by loops (i.e., amino acid 11-17 loop, receptor recognition motif) and turns (e.g., 30-35 turn), three antiparallel β-strands, and an overlying COOH-terminal α-helix. Reproduced with minor modification from Rojo et al. (Rojo, Suetomi et al. 1999).

1.2.3.4. CXCR1 and CXCR2 signaling

IL-8 binds two distinct receptors, the CXCR1 and CXCR2, which comprise 350 and 360 amino acids, respectively, and possess >77% sequence homology at the amino acid level (Baggiolini, Dewald et al. 1997). They are intramembranous GPCRs composed of seven-transmembrane domains (Mukaida 2003). High resolution structural data for CXCR1 and CXCR2 have not been generated yet, but studies have shown that the N-domain plays an important role in ligand binding (Suzuki, Prado et al. 1994), although its role in determining receptor selectivity is still not clear. Similar to that of other ELR-CXC chemokines, IL-8-stimulated CXCR1 or CXCR2 signaling consists of two steps (Figure 1.2.). First, the IL-8 N-terminal loop, surface hydrophobic pocket and/or adjacent residues cooperate and selectively interact with N-terminal domain residues of the receptors (site I). Second, the IL-8 N-terminus, including the ELR motif and Ile^{10} (site II), interacts with the receptor exoloop and transmembrane (J-domain) residues, which signals into and activates the cell (Schraufstatter, Ma et al. 1995; Hammond, Shyamala et al. 1996; Wells, Power et al. 1996; Suetomi, Lu et al. 1999; Rajagopalan and Rajarathnam 2004).

These interactions mediate ligand affinity, receptor selectivity, and cell activation. Whether binding at one site influences binding/function at the other site is still not clear (Rajagopalan and Rajarathnam 2004).

1.2.3.5. CXCR1 and CXCR2 internalization and desensitization

During inflammation, neutrophils receive multiple signals from inflamed tissues. Neutrophil chemoattractants such as IL-8, fMLP, C5a, PAF, and LTB4, are important signals that chemoattract neutrophil to the inflammation sites. They all bind their respective GPCRs on the cell surface to induce cellular responses via G protein and phopholipase C activation. In order to facilitate a single modified response in the face of exposure to multiple overlapping ligands, neutrophils can display differential GPCR desensitization (Blackwood, Hartiala et al. 1996; Ali, Richardson et al. 1999). Two types of desensitization have been noted: homologous and heterologous. Homologous desensitization occurs when receptor is occupied and becomes hyporesponsive to subsequent agonist stimulation through a process involving phospholation of GPCR kinase

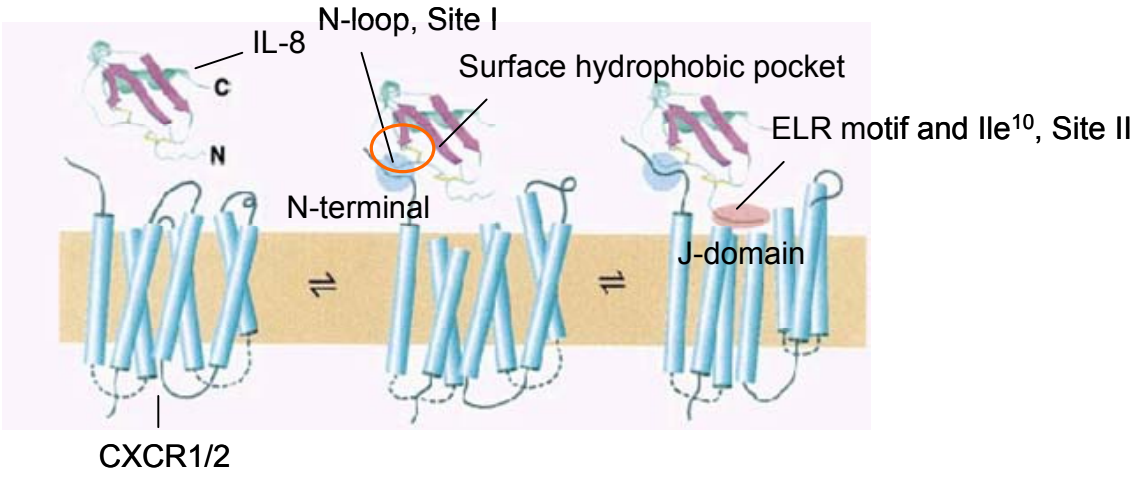


Figure 1.2. The model of the interaction between IL-8 and CXR1 or CXCR2.

IL-8 activatation of the CXCR1 or CXCR2 may consist of two steps. First, the IL-8 N-terminal loop, surface hydrophobic pocket and /or adjacent residues cooperatively interact with N-terminal residues of the receptor. Second, the IL-8 N-terminus, including the ELR motif and Ile^{10} interact with the receptors' first and second extracellular loops and transmembrane (J-domain) residues, which induces signaling into and activation of the cells. Edited from (Baggiolini 2001).

(GRKs). Heterologous desensitization describes a process whereby activation of one type of receptor (e.g., the CXCR1) results in loss of responsiveness to a different GPCR ligand (e.g., C5a). This process does not require receptor occupancy (i.e. of the second receptor) and involves phosphorylation of second messenger-activated kinases (e.g., protein kinase C; PKC) (Richardson, Ali et al. 1995; Ali, Richardson et al. 1999). Desensitized receptors can regain sensitivity in the absence of stimulation.

GPCR desensitization usually involves a functional uncoupling from G proteins, receptor internalization into the intracellular compartment, and receptor down-regulation. Using calcium flux assays in receptor-transfected cell lines have shown that IL-8 can heterologously desensitize receptors for fMLP and C5a through CXCR1, and *vice versa*. The CXCR2 does not take part in the cross-regulate responses to other chemoattractant receptors. IL-8 also can heterologously desensitize receptors for LTB4 and PAF, but not *vice versa*. Studies using neutrophil chemotaxis assays also demonstrated that fMLP, C5a, and IL-8 can heterologous desensitize each other and fMLP is most efficient at this (Ali, Richardson et al. 1999). However, IL-8 less potently induces heterologous desensitization than fMLP and C5a do between each other (Richardson, Pridgen et al. 1998; Ali, Richardson et al. 1999).

Generally, these studies indicate that there is a hierarchy among neutrophil GPCRs. The potency of receptors to heterologous desensitize one another is fMLP > C5a > IL-8 > LTB4, PAF. IL-8-mediated cross-desensitization is regulated via inhibition of phosholipase C (PLC) activation downstream of other groups of chemoattractant receptors (Ali, Richardson et al. 1999). Evidence suggests that this may be due to differences in signaling pathways whereby neutrophil responses to IL-8, PAF, and LTB4 are dependent on phosphoinositide-3-kinase (PI3K)/Akt pathway, whereas p38 mitogen-ativated kinase (p38 MAPK)-dependent signaling mainly mediates neutrophhil responses to fMLP and C5a. There is a hierarchy between the two pathways in which the former can be inhibited by the latter (Haribabu, Zhelev et al. 1999; Heit, Tavener et al. 2002). This inhibition may be necessary and important for neutrophils to reach the inflammation sites. During an infection, multiple chemoattractants are released in varied locations by resident cells such as endothelial cells, epithelial cells, and macrophages. Neutrophils from vasculature need to decide which chemoattractant to follow. Foreign bacterial peptide (e.g., fMLP) and the

bacterial product (e.g., LPS)-induced complement (e.g., C5a) may be more dangerous singals for the innate immunity. Therefore, neutrophils may have evolved such that the (fMLP- and C5a- activated) MAPK pathway dominates when faced with multiple chemoattractants. This decision-signaling process of neutrophils is crucial for the innate immunity to focus on the most dangerous targets, foreign pathogens. In addition, IL-8-CXCR1 signaling can heterologuous desensitization of C5aR and FPR may be because (1) IL-8 activated CXCR1 can phosphorylate FPR and C5aR with limited level resulting in decreased activation of PLC, (2) IL-8 activated CXCR1 downstream signaling, PI3K/Akt, may also interfere p38-MAPK phosphorylation activated by C5a and fMLP (see Fig 1.3.).

1.2.3.6. Roles of CXCL8, CXCR1 and CXCR2 in inflammation

CXCL8 was first purified as a chemotactic factor for neutrophils (Yoshimura, Matsushima et al. 1987). However, subsequent studies demonstrated that IL-8 exhibits multiple effects on neutrophils, including induction of shape change, release of lysosomal enzymes (Peveri, Walz et al. 1988), induction of a respiratory burst (Peveri, Walz et al. 1988), generation of superoxide, hydrogen peroxide (Thelen, Peveri et al. 1988), and bioactive lipids (Schroder 1989), and increases in the expression of adhesion molecules (Detmers, Lo et al. 1990). Moreover, IL-8 enhances transendothelial migration of these cells by inducing rapid shedding of L-selectin (CD62L) (Huber, Kunkel et al. 1991).

CXCR1 and CXCR2 have functionally distinct roles in inflammation. For instance, they are both important in mediating neutrophil chemotaxis, MPO and elastase release, and cell calcium mobilization (Jones, Wolf et al. 1996; Gonsiorek, Fan et al. 2007), but it is the CXCR1, and not the CXCR2, that mediates neutrophil respiratory burst and activation of phospholipase D (Jones, Wolf et al. 1996). On the other hand only the CXCR2 is important in mediating MMP-9 release from neutrophils (Chakrabarti and Patel 2005).

1.2.3.7. ELR-CXC chemokines in inflammatory diseases

The ELR-CXC chemokines are often expressed redundantly during inflammatory

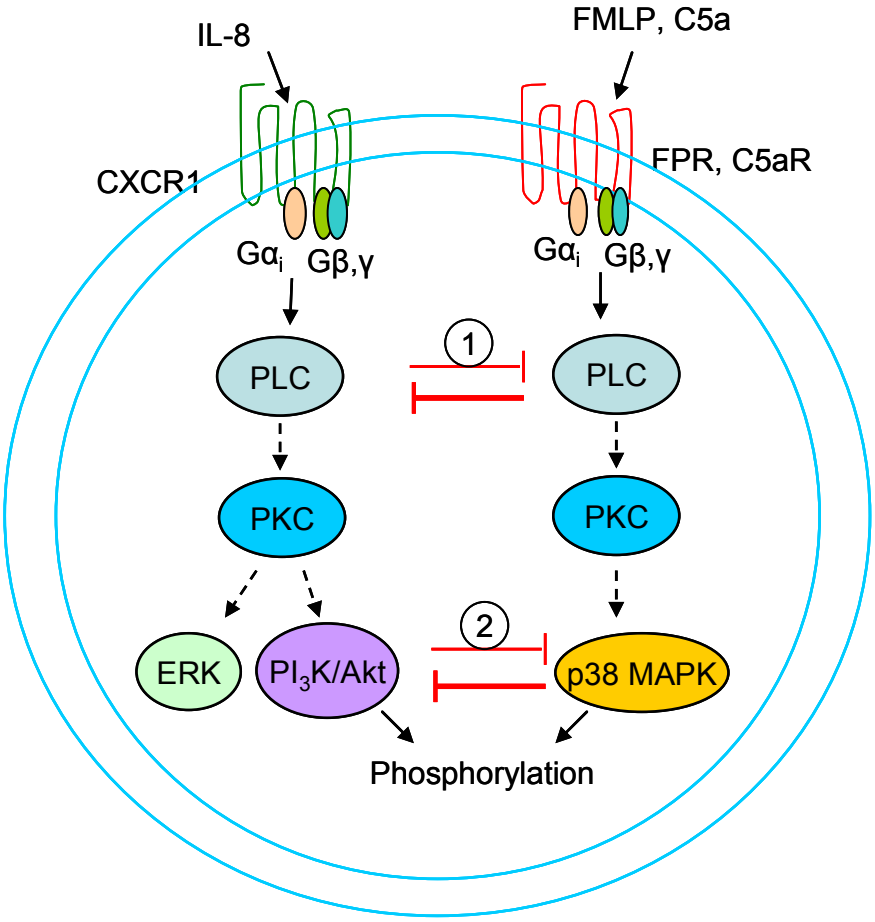


Figure 1.3. Cross-desensitization of neutrophil chemoattractant receptors.

Peptide chemoattractants (fMLP, C5a, IL-8) activate signaling pathways through $G\alpha_i$ and $\beta\gamma$ subunits to activate phosholipase C (PLC). PLC can further activate protein kinase C (PKC) and provide signals to cross-phosphorylate other chemoattractant receptors. FPR and C5aR are more resistant to the phosphorylation driven by CXCR1 signaling, while they are better able to phosphorylate thereby desensitize the CXCR1. ERK and PI3K are the down-stream signaling intermediates of CXCR1 signaling, while MAPK is the down-stream intermediate of FPR and C5aR.

responses (Miller, Cohen et al. 1992; Caswell, Middleton et al. 2001; Remick, Green et al. 2001), and often exacerbate the pathology of inflammatory diseases. Studies have demonstrated that ELR-CXC chemokines play an important role in mediating the pathology of ARDS (Strieter, Keane et al. 2005), wherein they mediate neutrophil recruitment and activation, a hallmark of ARDS (Abraham 2003). Elevated levels of IL-8 correlate highly with pulmonary neutrophilia and the mortality of ARDS (Miller, Cohen et al. 1992; Aggarwal, Baker et al. 2000). ELR-CXC chemokine-mediated neutrophilia in the respiratory system also greatly contributes to the severity of COPD pathology. High expression of IL-8, ENA-78 and their common receptor, CXCR2 has been demonstrated to be critical to the exacerbation of COPD (Qiu, Zhu et al. 2003). Ischemia and reperfusion of a local organ often causes remote organ injury, resulting in multiple organ dysfunction syndrome (MODS), and neutrophil activation is thought to be a primary reason of MODS. The severe pulmonary injury associated with MODS often results in ARDS (Carden and Granger 2000). ELR-CXC chemokines, e.g., IL-8, ENA-78, and CINC-1, are strongly upregulated in pulmonary, hepatic, skeletal, and intestinal ischemia and reperfusion injury models (Sekido, Mukaida et al. 1993; Colletti, Kunkel et al. 1995; Bless, Warner et al. 1999; Ishii, Ishibashi et al. 2000). By use of antibody neutralization approaches, it has been demonstrated that ELR-CXC chemokines have an important role in mediating neutrophil-induced injury (Sekido, Mukaida et al. 1993; Bless, Warner et al. 1999). IL-8 plays a crucial role in attracting neutrophils into synovial fluid and joint tissues in RA,where MMP-9 released by activated neutrophils contribute significantly to cartilage destruction (Van den Steen, Proost et al. 2002). Taken together, these results strongly suggest that strategies should be taken to block ELR-CXC chemokine-induced excessive neutrophil responses.

1.2.3.8. ELR-CXC chemokine antagonism and other anti-inflammatory therapies

1.2.3.8.1. Neutrophil antibody

As discussed above, ELR-CXC chemokines are often redundantly expressed during inflammatory responses, which results in excessive activation of neutrophils and exacerbation of pathology. The development of a means to effectively antagonize their

actions has been a clinical priority for some time (Harada, Mukaida et al. 1996; Baggiolini and Moser 1997). Since neutrophils are important effector cells in acute lung injury, anti-neutrophil antibodies have been used to improve the pathologies induced by hemorrhage, endotoxemia, and myocardial ischemia. These studies indicated that anti-neutrophil antibody treatments could decrease inflammatory cytokine (e.g., MIP-2, IL-1β, and/or TNFα) expression and the extent of lung edema in hemorrhage- or endotoxemia-induced lung injury models (Abraham, Carmody et al. 2000). In a myocardial ischemia/reperfusion (I/R) model, it has also been shown to improve survival rates of I/R animals, lower expression of IL-6 and IL-8, reduce myocardial neutrophil infiltration, and produce smaller myocardial infarcts as compared to the untreated control animals (Kohtani, Abe et al. 2002).

1.2.3.8.2. ELR-CXC chemokine antibodies and glucocorticoids

Anti-ELR-CXC chemokine antibodies, such as anti-IL-8, -MIP-2, -KC, and -CINC were developed to treat inflammatory diseases, especially I/R injury. They have been shown to be effective in ameliorating lung, intestinal, skeletal and renal I/R injury (Sekido, Mukaida et al. 1993; Bless, Warner et al. 1999; Miura, Fu et al. 2001; Souza, Bertini et al. 2004). Since different ELR-CXC chemokines, e.g., ENA-78 in COPD (Qiu, Zhu et al. 2003), may play redundant roles in inflammation, it would be ideal to simultaneously block all ELR-CXC chemokine functions. It has been demonstrated that administration of antisera for both MIP-2 and KC before reperfusion of ischemic kidneys yields more effective inhibition of the pathology than either antiserum alone (Miura, Fu et al. 2001), which also support the notion that these chemokines have redundant functions. Glucocorticoids have been used as anti-inflammatory agents to reduce the exacerbation of diseases characterized by airway inflammation, such as asthma. The main effect of this drug is to inhibit cytokine (e.g., IL-8) gene transcription and expression (Mukaida, Okamoto et al. 1994; Zhang, Truong-Tran et al. 2007). It may also enhance innate immune responses in airway mucosa, especially epithelial cells (Zhang, Truong-Tran et al. 2007). Glucocorticoids have many side effects, such as immunosuppression, which impacts our abilities to use them extensively.

1.2.3.8.3. ELR-CXC chemokine receptor antibody and antagonists

As noted, the ELR-CXC chemokines activate neutrophils through binding to the CXCR2 or the CXCR1 and CXCR2. It is obvious that blockade of both receptors (as opposed to only one) could be a more effective approach to inhibiting target cell function. There are a couple of studies that have addressed a hexapeptide antileukinate (Ac-RRWWCR-NH2) CXCR2 antagonist which has been used to treat inflammatory diseases. The data from these two studies indicated that antileukinate has potent abilities to inhibit neutrophil influx and cytokine and chemokine expression in lung tissues during hemorrhage-primed, cecal ligation and puncture-induced, acute lung injury (Lomas-Neira, Chung et al. 2004). This CXCR2 antagonist is also effective in protecting mice against acute pancreatitis and associated lung injury, which is believed to be mediated by ELR-CXC chemokines (Bhatia and Hegde 2007). Another type of CXCR2 angonist is a series of non-peptide antagonists including SB225002 and SB256610. SB225002 is a synthetic chemical that competitively binds CXCR2 on neutrophils and blocks IL-8-induced neutrophil chemotaxis *in vitro* and neutrophil margination *in vivo* (White, Lee et al. 1998). SB265610 can antagonize neutrophil calcium mobilization and chemotaxis-induced by CINC-1, and block pulmonary neutrophil accumulation induced by hyperoxia (Auten, Richardson et al. 2001). Another synthetic chemical, Sch527123, is able to block CXCR2-mediated neutrophil chemotaxis and to antagonize neutrophil infiltration and goblet cell hyperplasis in LPS-challenged rat lungs. These drugs are mainly focused on CXCR2-mediated inflammatory responses. As discussed previously, neutrophil release of ROI and elastase are mainly mediated via the CXCR1 (Jones, Wolf et al. 1996; Jones, Dewald et al. 1997), such that it would be ideal to incorporate blockade of both CXCR1 and CXCR2 in anti-inflammatory therapies. A class of derivatives of 2-arylphenylpropionic acid, including repertaxin and DF2162, are reported to be effective, non-competitive, allosteric inhibitors of the chemokine receptors CXCR1 and CXCR2. These two molecules are able to block neutrophil chemotaxis, calcium mobilization-induced by IL-8, CINC-1, or/and GROα. They are also able to prevent intestinal I/R injury and adjuvant-induced arthritis in rats (Souza, Bertini et al. 2004; Barsante, Cunha et al. 2008). But in these studies, there is no unequivocal evidence to show that these two drugs target CXCR1-mediated functions,

e.g., neutrophil ROI release, so it is hard to conclude that they antagonize both the CXCR1 and CXCR2.

Early studies have shown that minimal amino-terminal truncation of CXCL5 (Nufer, Corbett et al. 1999), CXCL6 (Wuyts, D'Haese et al. 1999) or CXCL8 (Clark Lewis, Dewald et al. 1994) significantly increases their respective receptor affinities. Natural amino truncation of CXCL8 potentiates its CXCR1-, but not CXCR2-, dependent activities by 10- to 27-fold (Van den Steen, Proost et al. 2000). Truncation into, but not beyond, the receptor-signaling ELR motif of some CXC chemokines can transform them into receptor antagonists (Moser, Dewald et al. 1993). But truncated CXCR2 ligands do not fully block CXCL8 signaling and ELR-truncated CXCL8 displays a relatively low receptor affinity (Moser, Dewald et al. 1993; McColl and Clark Lewis 1999). Truncation of the 5' terminal two amino acids of bovine CXCL8, combined with a lysine to arginine substitution at amino acid 11 (i.e., CXCL8 $_{(3\text{-}73)}$ K11R), is associated with significantly higher affinities for both CXCR1 and CXCR2, thus creating a CXCL8 analogue that possesses remarkably strong neutrophil agonist activities (Li and Gordon 2001). By use of two additional amino acid substitutions, G31P or P32G (Li and Gordon 2002), both shown by Clark-Lewis and colleagues to be important for CXCL8 activity (Clark Lewis, Dewald et al. 1994), our lab developed another two mutants, CXCL8$_{(3\text{-}73)}$K11R/G31P and CXCL8$_{(3\text{-}73)}$K11R/ P32G, which both had strong antagonist activities. Additional substitutions within these two candidate antagonists were all within the reported receptor recognition region of the molecule (i.e., R11, T12, H13, K17) (Li and Gordon 2002), such that we had generated two antagonist families (i.e. G31P- and P32G- based). Among them, bovine CXCL8$_{(3\text{-}73)}$K11R/G31P (bG31P) was superior to all others (Li and Gordon 2002). bG31P blocks binding of monospecific anti-CXCR1 and anti-CXCR2 antibodies to neutrophils (Li and Gordon 2002) and, when titrated against its parent chemokine, 0.5 nM bG31P 50% blocks neutrophil responses to 129 nM (1 μg/ml) CXCL8 (Li, Zhang et al. 2002). At ≤10 ng/ml, it also 93-97% blocks responses to the neutrophil chemoattractants present in wash fluids from the lungs of animals with clinical pneumonic pasteurellosis (Li, Zhang et al. 2002). Our lab has also confirmed the efficacy (i.e., up to 98%) of bG31P in blocking neutrophil infiltration of endotoxin challenge sites in cattle and it does so for 2-3 dy following a single injection (Li, Zhang et al. 2002). So, bG31P is a potent ELR-CXC chemokine

antagonist. Furthermore, we have evidence that bG31P can strongly antagonize agonist activity of human CXCL8 for human neutrophils.

CHAPTER 2 : RATIONALE, HYPOTHESIS, AND OBJECTIVES

Rationale

In developing $CXCL8_{(3-74)}$K11R/G31P (bG31P), our lab had first generated an exceptionally high binding affinity analogue of bovine CXCL8 analogue, $CXCL8_{(3-74)}$K11R. This analogue had ~ 450 fold greater binding affinity for neutrophils than did wild-type bovine CXCL8 (Li and Gordon 2001). We used this high affinity analogue to subsequently generate bG31P, which was shown to be highly effective in blocking ELR-CXC chemokine-induced neutrophil responses *in vitro* and neutrophilic pathology *in vivo* (Li and Gordon 2002; Li, Zhang et al. 2002; Gordon, Li et al. 2005). When we began to examine the issue of generating a fully human orthologue of bG31P, we looked at the human CXCL8 structure-function studies by Ian Clark-Lewis' lab, wherein it was reported that human $CXCL8_{(3-72)}$K11R has an only 4-fold greater binding affinity for neutrophils than its parent molecule, human $CXCL8_{(3-72),}$. This raised a question in our minds as to whether a human $CXCL8_{(2-72)}$K11R-based form of a human G31P would be of sufficiently high affinity to be a clinically useful competitive antagonist. In addition, Clark-Lewis had reported that human $CXCL8_{(4-72)}$G31P/P32G had a 20-fold reduced binding affinity for neutrophils relative to wild-type CXCL8 (Clark Lewis, Dewald et al. 1994), and this raised further questions in our minds regarding a purely human form of G31P. As a result, we elected to take a two-pronged approach to generating a human(ized) G31P, including humanization of the bovine molecule, as described in detail below, and simply making and testing a fully human form. My part of this included generating and characterizing humanized forms of the drug, and characterizing the human G31P that had been generated by others in the lab. Therefore, based on the following information:

- bG31P is a potent bovine ELR-CXC chemokine antagonist;
- bG31P can strongly antagonize human CXCL8 agonist activity for human neutrophils;

- bovine and human CXCL8 share relatively high amino acid sequence identity; and
- Most of discrepant amino acids between bovine and human CXCL8 are located in the carboxyl-terminal half of the molecule and knowledge that several amino-terminal residues (e.g., amino acids 4-6, 10-22, and 30-35) are critical for receptor binding and activation,

My hypothesis is that:

A humanized form of bG31P, or human G31P, can also block the actions of ELR-CXC chemokines on neutrophils. This novel antagonist should provide an effective anti-inflammatory therapy for ELR-CXC chemokine-driven neutrophil-mediated pathology.

My objectives were to:

1) Develop a humanized form of the ELR-CXC chemokine antagonist, bovine $CXCL8_{(3-74)}$K11R/G31P and assess its antagonist activities *in vitro* and *in vivo*.
2) Characterize a fully human form of the ELR-CXC chemokine antagonist, bovine $CXCL8_{(3-74)}$K11R/G31P (generated by others in the lab) using *in vitro* assays and a guinea pig airway endotoxemia model.
3) Test the role of human $CXCL8_{(3-72)}$K11R/G31P (hG31P) in blocking aspiration of gastric content-induced bacterial (i.e., aspration) pneumonia.
4) Examine the effect of hG31P on intestinal ischemia and reperfusion-induced neutrophilic pathology.

CHAPTER 3 : HUMANIZED FORMS OF THE CXCR1/CXCR2 ANTAGONIST, BOVINE CXCL8$_{(3–74)}$K11R/G31P, EFFECTIVELY BLOCK ELR–CXC CHEMOKINES ACTIVITY AND AIRWAY ENDOTOXEMIA PATHOLOGY [1]

Chapter 3 overview

Based on a review of the theoretical literature regarding the structure-activity relationships within the ELR-CXC chemokine CXCL8, we had anticipated that a human analogue of bG31P, CXCL8$_{(3-72)}$K11R/G31P (i.e., hG31P), may not be a potent antagonist for ELR-CXC chemokines, as noted above (Chapter 2 Rationale). So, the first step of my dissertation research was to humanize bG31P. The manuscript comprising this chapter describes the rationale and approaches for generating and characterizing a chimeric humanized form of bG31P, human-bovine (hb) G31P, and its analogues. Since human CXCL8 and bovine CXCL8 share high homology and the structure and function study showed that NH2-terminus is biologically and structurally important, I developed a chimeric protein, bCXCL8$_{(3-44)}$K11R/G31P-hCXCL8$_{(45-72)}$ (i.e., hbG31P). I subsequently used the vector for this hbG31P as a target for site-directed mutagenesis and generated T3K, H13Y, T15K, E35A, and S37T substitutions within hbG31P. I assessed the antagonist activities of these proteins using neutrophil chemotaxis, calcium flux and WBC ROI release assays. I also examined the abilities of hbG31P to block neutrophil infiltration in a guinea pig airway endotoxemia model. In this study, I designed and carried out all the experiments, although I had assistance from other co-authors in the *in vitro* and *in vivo* experiments. I analyzed the data and co-wrote the manuscript with Dr. Gordon.

[1] Zhao Xixing, F. Li, J. R. Town, X. Zhang, W. Wang, and J.R. Gordon 2007. Humanized forms of the CXCR1/CXCR2 antagonist, bovine CXCL8$_{(3–74)}$K11R/G31P, effectively block ELR–CXC chemokine activity and airway endotoxemia pathology. *International Immunopharmacology 7: 1723–1731*

3.1. INTRODUCTION

The ELR-CXC chemokines, so named because of their amino sub-terminal Glu-Leu-Arg (ELR) and Cys-X-Cys motifs, are important for neutrophil recruitment. CXCL8 (i.e., IL-8), the prototypical ELR-CXC chemokine, binds to the CXCR1 and CXCR2 receptors with high affinity, whereas all others, including GRO-α, -β, and -γ, ENA-78, GCP-2, and NAP-2 (i.e., CXCL1-3 and 5-7, respectively) have lower affinities, primarily for the CXCR2 (Baggiolini 1998). Their expression is up-regulated during inflammatory events (e.g., acute respiratory distress syndrome, inflammatory bowel disease; (Baggiolini, Dewald et al. 1994; Baggiolini, Dewald et al. 1997)), so that they have been broadly implicated in the pathology of many inflammatory diseases (Cummings, Martin et al. 1999; Mehrad, Strieter et al. 1999). Numerous other mediators (e.g., C5a, LTB4) are also relevant to such diseases of course (Tsai, Strieter et al. 2000; Woodruff, Arumugam et al. 2003; Chen, Lam et al. 2006), but because of their pathogenic roles in multiple settings, the development of specific ELR-CXC chemokine inhibitors has become an important research goal (Carvalho, Wakabayashi et al. 1997; Ramos, Fernandes et al. 2006).

We previously generated a very high affinity analogue of bovine CXCL8, $CXCL8_{(3–74)}$K11R (Li and Gordon 2001), which we used to generate a similarly high affinity, broad-spectrum ELR-CXC chemokine antagonist, bovine $CXCL8_{(3–74)}$K11R/G31P (bG31P). This antagonist blocks the abilities of ELR-CXC chemokines to activate or chemoattract neutrophils *in vitro* (Li, Zhang et al. 2002) and *in vivo* (Li, Zhang et al. 2002). A single treatment with bG31P ≈ 97% blocks neutrophil infiltration into intradermal endotoxin challenge sites for 2-3 days (Li, Zhang et al. 2002), and dramatically reduces pulmonary pathology and pyrexia in endotoxemic animals (Gordon, Li et al. 2005). While bG31P could perhaps be useful in the humans, it goes without saying that a human equivalent thereof would be of significantly more use. However, prior structure-function data has indicated that human $CXCL8_{(3-72)}$K11R is not a particularly high affinity neutrophil agonist, and that a G31P substitution within human $CXCL8_{(3-72)}$ only marginally reduces its neutrophil agonist activities (Clark Lewis, Dewald et al. 1994), suggesting that human $CXCL8_{(3-72)}$K11R/G31P (i.e., the human counterpart of bG31P) would not be a particularly

effective chemokine antagonist. Thus, we began a systematic humanization of bG31P to determine whether partially humanized bG31P isoforms might still be effective ELR-CXC chemokine antagonists.

Bovine and human CXCL8 share the homology of 78% at amino acid level. Most of the discrepant amino acids reside in the carboxy-terminal half of the molecule (Table 3.1.), while the amino-terminal residues (e.g., amino acids 4-6, 10-22, and 30-35) are essential for receptor binding and activation (Clark-Lewis, Schumacher et al. 1991; Clark Lewis, Dewald et al. 1994; Hammond, Shyamala et al. 1996). Thus, we first ligated the amino and carboxy portions of bG31P and human CXCL8, respectively, to generate bCXCL8 $_{(3-44)}$ K11R/G31P-hCXCL8 $_{(45-72)}$ (i.e., hbG31P), and found that it fully retained the antagonist activity of bG31P for human neutrophils. We then one-by-one substituted the remaining human-bovine discrepant amino acids (i.e., T3/K, H13/Y, H15/K, E35/A, and S37/T), and expressed and characterized each of these further humanized hbG31P analogues.

3.2. MATERIALS AND METHODS

3.2.1. Reagents and supplies

We have reported most reagents employed (Li and Gordon 2001; Li and Gordon 2002; Li, Zhang et al. 2002; Gordon, Li et al. 2005), but in addition we purchased: fluo-4 AM (Invitrogen Inc, Burlington, ON); QIAprep Spin Miniprep (QIAGEN Inc., Mississauga, ON); QuickChange^{TM} Site-Directed Mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA); hexadecyltrimethylammonium bromide (HTAB), tetramethylbenzidine (TMB), luminol, (Sigma Chemical Co, Mississauga, ON); biotinylated anti-human CXCL8 antibody (R & D Systems, Minneapolis, MN); and rabbit anti-human lactoferrin antibody, recombinant (r) lactoferrin and biotinylated anti-lactoferrin Ab (ICN Biomedicals Inc., Irvine, CA). Hartley guinea pigs (5 week-old females) were purchased from Charles River Laboratories (Charles River, MA); all experiments were carried out in accord with the guidelines of the Canada Council on Animal Care.

Table 3.1. The amino acid sequences of bovine and human CXCL8/IL-8

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
bovine	**M**	***S***	***T***	E	L	R	C	Q	C	I	K	T	***H***	S	***T***	P	F	H	P	K	F	I	K	E	L
human	*S*	*A*	*K*	E	L	R	C	Q	C	I	K	T	*Y*	S	*K*	P	F	H	P	K	F	I	K	E	L

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
bovine	R	V	I	E	S	G	P	H	C	***E***	N	***S***	E	I	I	V	K	L	T	N	G	N	E	V	C
human	R	V	I	E	S	G	P	H	C	*A*	N	*T*	E	I	I	V	K	L	S	D	G	R	E	L	C

	51	52	53	54	55	5 6	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
bovine	L	N	P	K	E	K	W	V	Q	K	V	V	Q	V	F	V	K	R	A	E	K	Q	D	P	
human	L	D	P	K	E	N	W	V	Q	R	V	V	E	K	F	L	K	R	A	E	N	S			

Bovine and human CXCL8/IL-8 share 78% amino acid sequence homology, with most of the discrepant amino acids residing in the carboxy half of the molecule. The target residues for this study (i.e., amino acids, 3, 13, 15, 35, and 37) are bold and italicized.

3.2.2. Generation of recombinant analogues of bCXCL8$_{(3-74)}$K11R/G31P

A human CXCL8$_{(3-72)}$ cDNA was synthesized commercially (Takara Biotech, Dalian, China), such that the coding sequence was flanked by a 5'BamH I site and a 3' stop codon (TAA) followed by an EcoR I site, and ligated into the plasmid pGEX-2T to generate pGEX- hCXCL8$_{(3-72)}$. We previously reported the expression vector for bG31P, pGEX-bCXCL8$_{(3-74)}$K11R/G31P (Li, Zhang et al. 2002). To generate a chimeric human-bovine form of bG31P, we digested the bovine and human plasmids pGEX-bCXCL8$_{(3-74)}$K11R/G31P and pGEX-hCXCL8$_{(3-72)}$, respectively, with Hind III and Pst I. We ligated the 1049 base pair Hind III/PstI fragment from pGEX-hCXCL8$_{(3-72)}$ (i.e., encoding hCXCL8 amino acids 44-72) into the 4100 base pair fragment from the digested pGEX-bCXCL8$_{(3-74)}$K11R/G31P (i.e., the bulk of the pGEX vector and the coding sequence for bG31P amino acids 3-43) to generate bCXCL8$_{(3-44)}$K11R/G31P-hCXCL8$_{(45-72)}$, or hbG31P. We used this chimeric vector as a target for site-directed mutagenesis (primers listed in Table 3.2.) to make T3K, H13Y, T15K, E35A, and S37T substitutions within the hbG31P vector. We verified all sequences commercially (Plant Biotechnology Institute, Saskatoon), then expressed and characterized each analogue by SDS-PAGE and Western blotting, as noted (Li and Gordon 2001; Li and Gordon 2002). All were the correct molecular weight (i.e., one band of ≈8 kD) and reacted with anti-human IL-8 antibodies.

3.2.3. Neutrophil chemotaxis assay

Our neutrophil isolation and modified Boyden chamber microchemotaxis assays have been described previously (Li and Gordon 2001; Li and Gordon 2002; Li, Zhang et al. 2002). Purified neutrophils were suspended in PBS^+ (phosphate-buffered saline [PBS; pH 7.4], 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 5 mM glucose, and 0.1% bovine serum albumin). The chemoattractants (e.g., CXCL8) and putative antagonists were mixed together for the assays, which were quantified by direct

Table 3.2. Site-directed mutagenesis PCR primers employed for the generation of hbG31P's mutants

Analogue	Upstream primers (5'-3' orientation)	Downstream primers (5'-3' orientation)
hIL-8(3-72)	C GGA TCC AAA GAA CTT AGA TGT CAG	G GAA TTC TTA TGA ATT TTC AGC CCT CTTC
hbG31P/T3K	CTG GTT CCG CGT GGA TCC AAA GAA CTT CGA TGC C	G GCA TCG AAG TTC TTT GGA TCC ACG CGG AAC CAG
hbG31P/H13Y	C CAA TGC ATA AGA ACA TAT TCC ACA CCT TTC C	G GAA AGG TGT GGA ATA TGT TCT TAT GCA TTG G
hbG31P/T15K	TGC ATA AGA ACA CAT TCC AAA CCT TTC CAC CCC	GGG GTG GAA AGG TTT GGA ATG TGT TCT TAT GCA
hbG31P/E35A	GT CCG CCA CAC TGT GCC AAT TCA GAA ATC	GAT TTC TGA ATT GGC ACA GTG TGG CGG AC
hbG31P/S37T	CCG CCA CAC TGT GAA AAT ACA GAA ATC ATT G	C AAT GAT TTC TGT ATT TTC ACA GTG TGG CGG

counting of at least five 40× objective fields within the stained chemotaxis assay membranes. In preliminary experiments optimal chemotaxis was induced with 10 ng/ml CXCL8. The results are expressed as the mean number of cells per 40× field ± SEM.

3.2.4. Reactive Oxygen Intermediate (ROI) release assay

To generate total white blood cells (WBC), heparin-anticoagulated human blood was mixed at a 1:1 ratio with 6% dextran (75 kDa) in PBS and allowed to stand undisturbed at room temperature until the red blood cells had sedimented. The WBC in the upper phase were washed and resuspended at 2.5×10^6 cells/ml in HBSS. For the assays, we mixed 50 µl of WBC, 25 µl of human CXCL8 (100 ng/ml) or HBSS, 25 µl of antagonist (containing 10, 50, 100 ng/ml hbG31P, final concentration) or HBSS, and 50 µl of Luminol (0.3 mM). In preliminary dose-response experiments, we had determined that optimal ROI release was induced with 100 ng/ml CXCL8. The ROI release was assessed by chemiluminescence using a microplate spectrofluorometer (NovoStar, BMG LABTECH Inc., Durham, NC). The data are expressed as the maximum sample luminescence of the experimental sample, minus the HBSS control values, in luminescence units.

3.2.5. Intracellular Ca^{2+} flux assay

Neutrophils (5×10^6 cells/ml in Ca^{2+}-free PBS^+) were stained for 30 min at 37˚C with 2 µM fluo-4 AM, washed twice with Ca^{2+}-free PBS^+ medium, resuspended at 4×10^6 cells/ml in PBS^+ containing 3.3 nM Ca^{2+}, and then kept at room temperature. For the assays, 50 µl of cells were preincubated for 15 min with 50 µl of medium or antagonist, and then challenged with 50 µl of medium or agonist (CXCL8 or CXCL1). Intracellular Ca^{2+} mobilization was assessed using a microplate spectrofluorometer (emission and excitation wavelengths, 520 nm and 488 nm, respectively) and based on so-generated ‘area under the curve’ readouts. Preliminary experiments showed that

100 ng/ml CXCL8 induced optimal PMN intracellular Ca^{2+} mobilization. For technical reasons associated with the high inter-experiment variance of spectrofluorimeter readouts, the data are expressed as the mean percent inhibition (± SEM) of agonist-induced Ca^{2+} flux across at least three independent experiments.

3.2.6. Guinea pig acute lung endotoxemia

Our guinea pig airway endotoxemia model has been noted previously (Gordon, Li et al. 2005). Briefly, 30 min before airway challenge with 200 µl of *Escherichia coli* LPS (serotype 0127B8**;** 5 µg/kg in PBS), the animals (n = 5) were given 250 µg/kg hbG31P or saline (0.5ml) s.c., then euthanized 15 h later with halothane. We had run preliminary hbG31P dose-response experiments using hbG31P doses of 50, 100 and 250 µg/kg and found that at the lower two doses we achieved only 20-40% inhibition of airway neutrophilia (data not shown). Bronchoalveolar lavage (BAL) fluid collection, BAL WBC (and RBC) enumeration and differentials were performed as noted (Gordon, Li et al. 2005); the results are expressed as the mean number of cells per BAL sample. Peripheral blood total WBC counts were also assessed. All samples were assessed independently.

3.2.7. Myeloperoxidase assay (MPO)

BAL fluids (10 µl) were diluted 10-fold with 0.5% HTAB, and then 10 µl of these diluted samples were incubated at room temperature for 10 min with 100 µl TMB, before stopping the reactions with 100 µl of 1M phosphoric acid. The reactions were quantified by spectroscopy (OD, 450 nm) and the data expressed as OD_{450} ± SEM.

3.2.8. Lactoferrin (LF) ELISA

We employed a standard capture ELISA format, with optimized concentrations of capture (2.45 µg/ml) and detection (0.85µg/ml) antibodies, as noted (Schneider, Li et al. 2001). The standards comprised lactoferrin (6.25- 400 ng/ml) and the samples

were diluted 1:10 for the assay. The results were expressed as the mean concentration (ng/ml) of each group ± SEM.

3.2.9. Lung Pathology

We euthanized the animals with halothane, then did bronchalveolar lavages (BAL) on each, after which we removed, photographed, and assessed the cardiopulmonary tree for pleural hemorrhagic consolidation. The caudal left lung lobe of each animal was fixed for 3 h in acid-alcohol formaldehyde and routinely processed to paraffin sections in our institutional pathology department. For routine histopathology, the tissue sections were stained with a standard hemotoxylin and eosin (H&E) protocol and examined in a blinded manner at 400× magnification. The histologic sections were photographed at 400× or 1000× magnification.

3.2.10. Statistical analysis

Two-group comparisons were made using student's t-tests (two-tailed). The results are expressed as the mean ± SEM.

3.3. RESULTS

3.3.1. hbG31P antagonizes human neutrophil chemotaxis, ROI release, and intracellular Ca^{2+} flux in response to CXCL8 and CXCL1 signaling.

The agonist activity of hbG31P for human neutrophils was tested using chemotaxis and WBC ROI release assays. CXCL8 was a strong agonist for both chemotaxis (Fig. 3.1A) and ROI release (Fig. 3.1B). Neither hbG31P nor bG31P displayed discernible agonist activity when used at 10, 50 (Fig. 3.1), or 100 (data not shown) ng/ml. On the other hand, 10 or 50 ng/ml hbG31P effectively blocked neutrophil chemotaxis induced by 10ng/ml CXCL8 or WBC ROI release stimulated by 100 ng/ml CXCL8 (Fig. 3.2A & B, respectively), as well as intracellular Ca^{2+} flux induced by CXCL1 or CXCL8 (each at 100 ng/ml; Fig.3.2C).

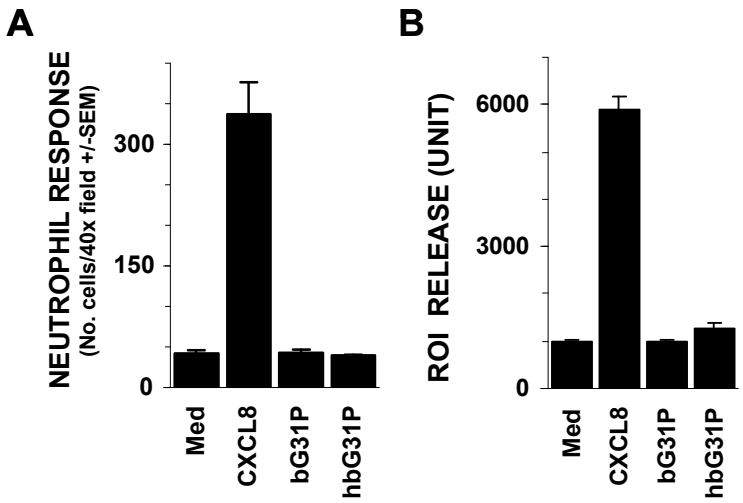


Figure 3.1. The human-bovine chimeric protein hbG31P is not a neutrophil agonist.

The agonist activities of hbG31P were compared with those of bG31P using chemotaxis and reactive oxygen intermediate (ROI) release assays (see material and methods). (A) The ability of hbG31P to chemoattract human neutrophils was compared to that of medium (Med.) alone, human CXCL8, and bG31P (each, 10 ng/ml). The results are expressed as the mean (±SEM) number of cells/40× objective microscope field. Neither hbG31P nor bG31P displayed discernible agonist activity relative to the medium control. (B) Similarly, the ability of hbG31P to induce ROI release from human WBC was compared to that of HBSS (med.), bG31P (each, 50 ng/ml) or human CXCL8 (100 ng/ml). Luminol-amplified luminescence was detected using a microplate spectrofluorimeter, and the data expressed as the maximum luminescence for each sample (± SEM). Neither bG31P nor hbG31P possessed significant neutrophil agonist activity when compared with medium alone. These results are representative of three independent experiments.

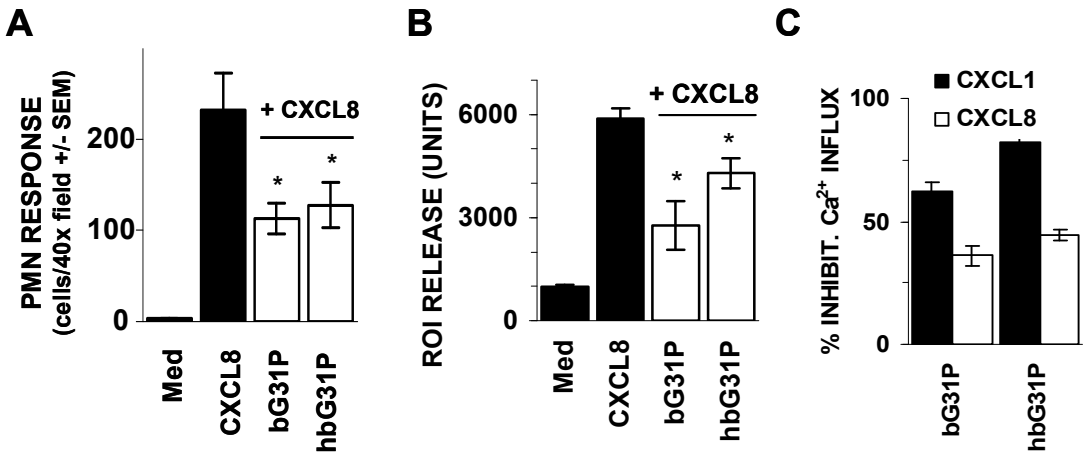


Figure 3.2. hbG31P is an effective ELR-CXC chemokine antagonist.

The antagonist activities of the hbG31P were compared with those of bG31P using chemotaxis and ROI release assays, as in Fig 3.1., as well as chemokine-induced intracellular Ca^{2+} flux, as noted in the methods. (A) Both bG31P and hbG31P (each, 10 ng/ml) inhibited human neutrophil chemotactic responses to CXCL8 (10 ng/ml; *, $p \leq 0.05$ versus CXCL8 alone) as well as (B) CXCL8-induced ROI release from human WBC (CXCL8, 100 ng/ml; bG31P or hbG31P, each 50 ng/ml, *, $p \leq 0.05$ versus CXCL8 alone). (C) Both antagonists (100 ng/ml) also blocked equimolar CXCL8- and CXCL1-induced neutrophil intracellular Ca^{2+} flux in human neutrophils; in 3 of 3 experiments hbG31P treatment led to ≈20% greater antagonism than did bG31P treatments. The percent inhibition by (h)bG31P of intracellular Ca^{2+} flux induced by CXCL1 and CXCL8 were calculated from the 'area under the curve' data of three independent experiments, as determined using Fluo-4 AM-loaded cells.

3.3.2. Further humanization of hbG31P does not reduce its antagonist activities.

Within hbG31P there remained five human-bovine discrepant amino acids (i.e., T3, H13, T15, E35, and S37; Table 3.1). Thus, we generated further hbG31P analogues in which each of these were replaced one-by-one (i.e., T3K, H13Y, T15K, E35A, and S37T) and confirmed that none of these were neutrophil agonists ($p>0.05$ versus medium alone; Fig. 3.3A). Furthermore, all except T15K strongly inhibited CXCL8-driven chemotaxis (Fig. 3.3B) and dose-dependently antagonized CXCL8-induced ROI release (i.e., versus 100 ng/ml CXCL8; Fig.3.3C). Furthermore, all were equally antagonistic for CXCL1- or CXCL8-induced intracellular Ca^{2+} flux except H13Y, whether tested at 10 (for each, ≈20% effective; data not shown) or 100 ng/ml (Fig. 3.3D). These results were representative of three experiments.

3.3.3. hbG31P is an effective antagonist of endotoxin-induced acute lung inflammation

Since hbG31P was confirmed to be a good antagonist *in vitro* and none of other analogues displayed markedly better antagonist activity (and some tended to be inferior), we confined our *in vivo* assessments to hbG31P, testing its activity in a standard guinea pig acute airway endotoxemia model, as described (Gordon, Li et al. 2005). As noted, at 15 h after induction, animals suffering from airway endotoxemia develop intense pulmonary inflammatory responses (Gordon, Li et al. 2005). The hbG31P treatments had protective effects as observed either grossly or histologically (Fig. 3.4A). At the gross pathology level, we observed marked hemorrhagic consolidation in the anterior right lung lobes of the saline-treated, LPS-challenged animals (LPS control), and treatment with 250 μg/kg hbG31P reduced this to near background (Fig. 3.4A, upper panels). When observed histologically, the inflammatory cell congestion in the lung tissues of the saline-treated LPS-challenged animals was very marked (Fig. 3.4A, lower LPS control panel), and most of these cells were neutrophils as determined by morphology and staining with H&E stains (inset). The hbG31P treatments dramatically reduced the inflammatory congestion in the lungs (Fig. 3.4A, lower right panel)

Figure 3.3. Further humanization of hbG31P does not markedly augment its antagonist activities.

We generated analogues of hbG31P in which each of the remaining human/bovine-discrepant amino acids were one-by-one replaced with their human equivalent residue, and then we compared each of these with hbG31P, as in Fig.3.1 and 3.2. (A) Neither hbG31P nor any of the analogues (10 ng/ml) displayed significant chemotactic activity relative to medium controls. These results are from one experiment that is representative of three independent experiments. (B) With the exception of T15K, each of the tested analogues (10 ng/ml) equally blocked CXCL8- (10 ng/ml) induced chemotactic responses (*, $p \leq 0.05$, **, $p \leq 0.01$, each compared to CXCL8 alone). (C) 1-100 ng/ml of each antagonist was used to characterize their activities in WBC ROI release assays. With the exception of H13Y, each analogue dose-dependently inhibited CXCL8-induced ROI release from stimulated WBC. At 100 ng/ml, each analogue equally blocked ROI release. (D) At 100 ng/ml, each analogue blocked intracellular Ca^{2+} flux in CXCL8- or CXCL1-stimulated neutrophils. None of these analogues displayed more potent antagonist activity than hbG31P.

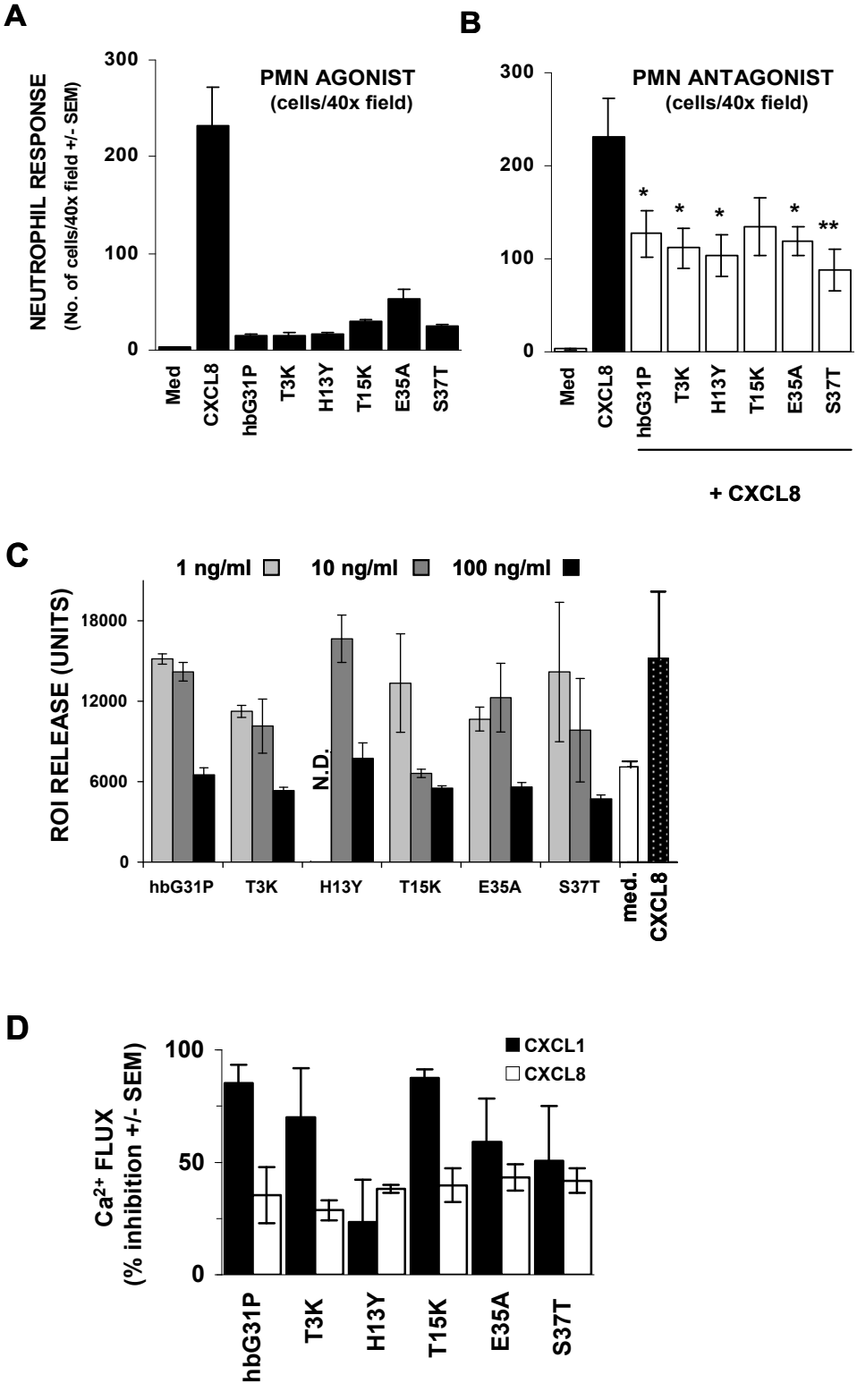
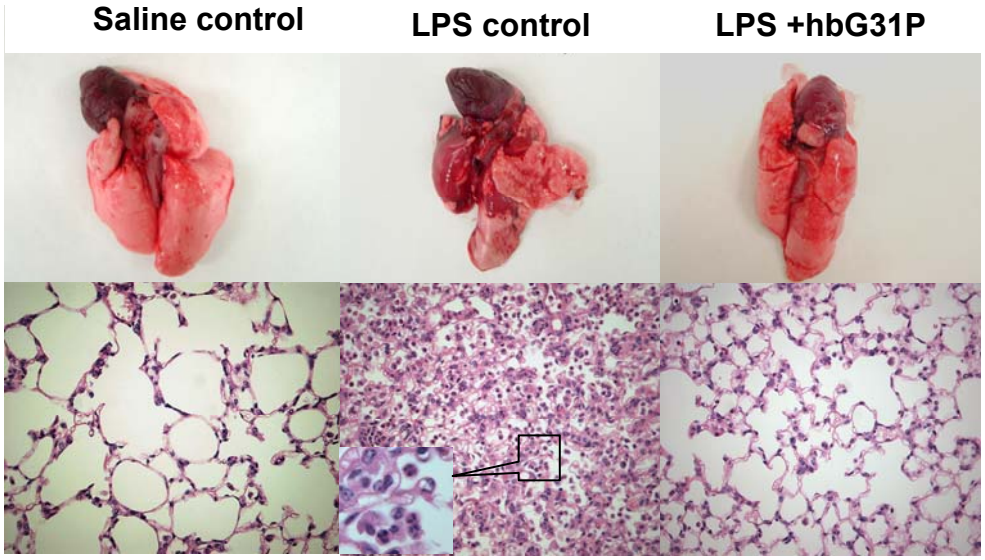

A
NEUTROPHIL RESPONSE
(No. of cells/40x field +/- SEM)
PMN AGONIST
(cells/40x field)
300
200
100
0
Med
CXCL8
hbG31P
T3K
H13Y
T15K
E35A
S37T
B
PMN ANTAGONIST
(cells/40x field)
*
**
+ CXCL8
C
1 ng/ml
10 ng/ml
100 ng/ml
ROI RELEASE (UNITS)
18000
12000
6000
0
N.D.
hbG31P
T3K
H13Y
T15K
E35A
S37T
med.
CXCL8
D
Ca2+ FLUX
(% inhibition +/- SEM)
CXCL1
CXCL8
100
50
0

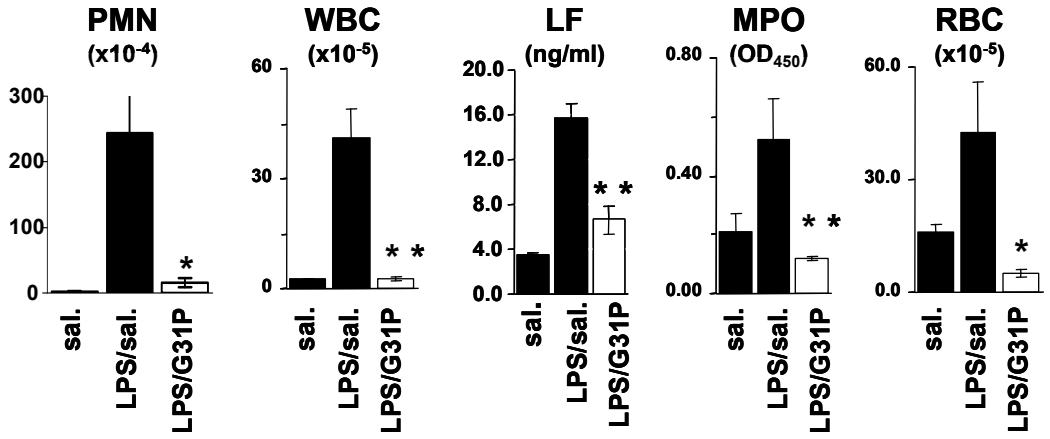
Figure 3.4. hbG31P displays potent anti-inflammatory activity in endotoxemic animals.

Airway endotoxemia was induced in guinea pigs by intranasal instillation of bacterial endotoxin (LPS; 5 μg/kg), while saline control animals were administered an equal volume of saline. The animals were treated 30 min before LPS challenge either with saline (LPS control) or hbG31P (LPS+hbG31P; 250 μg/kg), and euthanized at 15 h post-challenge. **(A)** The cardiopulmonary tree of each animal, including the heart (H) and lungs, was removed and photographed using a dorsal approach (upper panel), then processed to paraffin sections for H& E staining (lower panel). The lungs of the saline control animals were normal in gross and histological appearance (left panels), while the lungs of the LPS control animals, and particularly the cranial right lung lobe (circled) of each animal, were markedly hemorrhagic (middle panels) and congested with red blood cells RBC) and neutrophils (PMN; inset, LPS control histology). The lungs of the hbG31P-treated LPS-challenged animals were largely free of hemorrhagic consolidation and displayed greatly reduced pulmonary inflammation (right panels). **(B)** We performed bronchalveolar lavages (BAL) on each animal and the total neutrophil, WBC and RBC counts within the airways as noted in the materials and methods section. We also assessed the BAL fluid levels of myeloperoxidase (MPO) and lactoferrin (LF) by colorimetric assays and ELISA, respectively. The data indicate that hbG31P was highly effective in reducing PMN ($p \leq 0.05$ compared to saline therapy), white blood cell (WBC; $p \leq 0.05$), and RBC ($p \leq 0.01$) infiltration of the airways, and also in reducing the release of LF ($p < 0.01$) and MPO ($p < 0.01$) into this compartment.

A


Saline control
LPS control
LPS +hbG31P

B


PMN
(x10-4)
300
200
100
0
WBC
(x10-5)
60
30
0
LF
(ng/ml)
20.0
16.0
12.0
8.0
4.0
0.0
MPO
(OD450)
0.80
0.40
0.00
RBC
(x10-5)
60.0
30.0
0.0
sal.
LPS/sal.
LPS/G31P
*
* *

As expected based on our histological assessments, treatment with hbG31P reduced the numbers of neutrophils and total white blood cells (WBC) recoverable from the airways to near background ($p \leq 0.01$, versus saline-treated animals; Fig. 3.4B). Furthermore, the levels of the neutrophil degranulation markers MPO and LF in the BAL fluid of the hbG31P-treated animals were also dramatically lower than those in the saline-treated group. Thus, hbG31P was a highly effective antagonist of pulmonary neutrophil infiltration in endotoxemic animals. Furthermore, the numbers of red blood cells entering the airways following hbG31P-treatment were also dramatically reduced. This clearly confirmed that ELR-CXC chemokine antagonism can ameliorate loss of vascular integrity in endotoxemia, as noted previously (Gordon, Li et al. 2005). Thus, our data confirmed that overall the human-bovine chaemeric protein, hbG31P, has very potent anti-inflammatory activity, as determined in a robust model of acute lung inflammation.

3.4. DISCUSSION

We showed that the human-bovine chimeric protein bCXCL8$_{(3-44)}$K11R/G31P-hCXCL8$_{(45-72)}$ (i.e., hbG31P) fully retained the antagonist activity of bG31P. Among the ELR-CXC chemokines, the 'ELR' and the "11-17" loop (i.e., amino acids 11-17) motifs are critical for receptor signaling and recognition (Clark-Lewis, Dewald et al. 1994), respectively, while the COOH-terminal α-helix (e.g.,, amino acids 59-72) is important structurally, rather than for receptor binding (Clark-Lewis, Schumacher et al. 1991). Nevertheless, both the amino and carboxyl termini reportedly may be critical for CXCR2 binding (Schraufstatter, Barritt et al. 1993), while the hydrophobic pocket formed in part by Lys49, Tyr13, Ser14, and Phe21, also participates in CXCL8 receptor binding (Hammond, Shyamala et al. 1996). Our evidence indicates that the human-bovine discrepant amino acids within the carboxy half of bG31P (i.e., between T44 and P74) did not markedly influence its antagonist activities. It has been reported that neutrophil intracellular Ca^{2+} flux is mediated by both CXCR1 and CXCR2 signaling , while superoxide release is CXCR1-dependent (Jones, Wolf et al. 1996). Inasmuch as hbG31P impaired intracellular Ca^{2+} flux induced by CXCL1 (a CXCR2

ligand) and ROI release, our data suggests that hbG31P acts through both the CXCR1 and CXCR2, as was reported previously for bG31P (Gordon, Li et al. 2005).

Our data indicated that none of the human-bovine discrepant amino acids within hbG31P that we tested (i.e., T3, H13, T15, E35, and S37) markedly affected its antagonist activities. Thr3, the first amino acid within hbG31P, is proximal to both the receptor-signaling (i.e., the ELR) motif. Others have shown that residues proximal to the ELR motif are dispensable for activity among the ELR-CXC chemokines (Clark-Lewis, Schumacher et al. 1991). On the other hand, amino acids H13 and T15 both reside within the 'receptor recognition loop' of the molecule, a key region for binding to the CXCR1 (Schraufstatter, Ma et al. 1995), but we did not observe marked alterations in antagonist activities as a result of either H13Y or T15K substitutions. We knew that a non-conserved H13F substitution within the receptor recognition loop of bovine $CXCL8_{(3-73)}$K11R/G31P (i.e., bG31P) has little effect on the molecule's antagonist activity (Li, Zhang et al. 2002), although the conserved K11R substitution within bovine CXCL8 had a dramatic effect on receptor affinity (Li and Gordon 2001). Residues E35 and S37 reside just proximal to cysteine 34 (which sulfhydryl bonds directly into the ELR-CXC motif), and close to the junction of two β-pleated sheet regions of the molecule. Thus, perturbations in this region, with their potential to be translated across the C7-C34 cysteine bond into the vicinity of the ELR motif or to significantly affect the orientation of these β-pleated sheets, could potentially have had significant effects on the activity of hbG31P. But the non-conserved E35A substitution also did not significantly affect the molecule's antagonist activity *in vitro*, and this is in accord with the previous report that Ala35 of human CXCL8 is not an important functional determinant (Clark-Lewis, Dewald et al. 1994). The S37T substitution did yield a hbG31P analogue with slightly better antagonist activity against CXCL8-induced neutrophil chemotaxis and ROI release, although it did not seem to differentially affect intracellular Ca^{2+} flux in CXCL8-stimulated cells. Thus, it is possible that Thr37 of human CXCL8 may contribute modestly to the molecule's affinity for the CXCR1.

Acute endotoxin-induced lung injury is characterized by an accumulation of neutrophils in the airways (Togbe, Schnyder-Candrian et al. 2006), largely driven by

ELR-CXC chemokines (Cummings, Martin et al. 1999). Our data confirmed that hbG31P blocked total and neutrophilic leukocyte infiltration of the airways, as well as the pulmonary hemorrhagic response in this condition. The pulmonary neutrophil recruitment we observed would be related to ELR-CXC chemokine expression, but it could potentially also be affected by other mediators including, for example, LTB4, C5a (Tsai, Strieter et al. 2000; Woodruff, Arumugam et al. 2003; Chen, Lam et al. 2006), or PAF (Lad, Olson et al. 1985). Our antagonist blocked the ELR-CXC chemokines, but it is unlikely to directly affect the activities of such alternate agonists. The fact that hbG31P blocked ≈94% of the neutrophil infiltration of these endotoxemic airways raises the question of whether such alternate neutrophil chemoattractants are involved in this response and, if so, whether ELR-CXC chemokine antagonism in some way blocks these as well. This suggests that ELR-CXC chemokine antagonism has effects above and beyond blockade of high-level neutrophil infiltration of the tissues. For example, it could block the activities of the CXCR1 and/or CXCR2 expressed on resident lung cells (e.g., epithelial cells; (Farkas, Hahn et al. 2005)) and thereby the initiation of the ELR-CXC chemokine responses in the tissues. Since all neutrophil chemoattractants operate through G-protein coupled receptors (Murphy 1994), it remains to be seen whether G31P signaling has effects that go beyond simple blockade of the CXCR1 and CXCR2.

CHAPTER 4 : ELR-CXC CHEMOKINE RECEPTOR ANTAGONISM TARGETS INFLAMMATORY RESPONSES AT MULTIPLE LEVELS [2]

Chapter 4 overview

In Chapter 3, we reported that hbG31P and its analogues fully retained the antagonist activities of bG31P. To reach the final goal of developing a fully human form of CXCR1 and CXCR2 antagonist, recently, Ms Jennifer Town, Dr. Fang Li, and I generated a fully human form of bG31P from human CXCL8 (i.e., hG31P). Jennifer and I characterized the antagonist activities of this human protein in *in vitro* bioassays and *in vivo* models. They will be described in this chapter and also in Chapters -5,-6. In this chapter, we mainly assessed the antagonist activities of hG31P for ELR-CXC chemokine-induced neutrophil chemotaxis, ROI release, intracellular calcium flux, and apotosis. We also tested the antagonist activities of hG31P in blocking cystic fibrosis patient sputum-induced human neutrophil chemotaxis, and endotoxin-induced airway epithelial cell ELR-CXC chemokine and cytokine release. Moreover, we assessed the ability of hG31P to heterologously desensitize other GPCRs (e.g., C5aR, BLT1, FPR). Jennifer characterized several important effects of hG31P, e.g., heterologous desensitization of GPCR, the neutrophil apoptosis experiments, and epithelial cell cytokine and chemokine release. I was involved in setting up the neutrophil calcium flux and ROI release assays and examining the effects of the hG31P on neutrophil chemotaxis, calcium flux, and ROI release induced by ELR-CXC chemokines. I also undertook *in vivo* model system, guinea pig airway endotoxemia, to examine the antagonist activities of hG31P in acute lung injury. Finally, Jennifer and I were responsible for analyzing the data. Dr. Gordon and I co-wrote the manuscript with contributions from the other co-authors.

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[2] Zhao Xixing, J.R. Town, F. Li, X. Zhang, D.Cockcroft, and J.R. Gordon. ELR-CXC chemokine receptor antagonism targets inflammatory responses at multiple levels. 2009. *J Immunol. Mar 1; 182(5):3213-22.*

4.1. INTRODUCTION

Neutrophil responses are a critical element in host defense during bacterial infections, but can also be overtly pathogenic. Thus, during many inflammatory diseases neutrophils contribute more to the pathology than do the microbes themselves (Nathan 2002). Neutrophil recruitment is a complex process involving activation of local structural cells (e.g., epithelial cells) via pathogen-associated molecular pattern receptors, complement cascade products or arachidonic acid metabolites, such that the structural cells express inflammatory mediators (e.g., IL-1, CXCL8) (Smith, Fedyk et al. 2001). These can in turn activate regional endothelial cells, which actively foster intravascular neutrophil retention via the now classical paradigm of chemokine- (e.g., CXCL8) and adhesion molecule-mediated rolling-arrest-diapedesis (Springer 1994; Baggiolini 1998).

The ELR-CXC chemokines are a subgroup of the CXC chemokine family in which the amino sub-terminal two cysteine residues, which are separated by an alternate amino acid, are immediately preceded by a Glu-Leu-Arg motif. They include CXCL1-3 and 5-8 (growth-related oncogene α, β, and γ [GRO α, β, and γ], epithelial cell neutrophil-activating peptide-78 [ENA-78], granulocyte chemotactic protein-2 [GCP-2], neutrophil activating peptide-2 [NAP-2], and interleukin-8 [IL-8], respectively) (Baggiolini 1998), which chemoattract and activate neutrophils via two closely related G protein-coupled receptors (GPCR), the CXCR1 and CXCR2. CXCL8 binds both receptors with high affinity, while CXCL6 binds both receptors with lower affinity (Wolf, Delgado et al. 1998; Wuyts, Proost et al. 1998). The other ELR-CXC chemokines bind to the CXCR2, also with relatively lower affinities (Ahuja and Murphy 1996; Wuyts, Proost et al. 1998). Both the CXCR1 and CXCR2 can trigger chemotactic responses and intracellular Ca^{2+} flux in neutrophils and contribute to elastase release (Chuntharapai and Kim 1995; Wuyts, Proost et al. 1998), but activation of the respiratory burst and phospholipase D release responses are reportedly CXCR1-dependent (Jones, Wolf et al. 1996), whereas CXCR2 signaling is critical to MMP-9 release (Chakrabarti and Patel 2005). Moreover, these two receptors may be differentially involved in neutrophilic pathology *in vivo*. For example, the CXCR1 is reportedly of more importance in inflammatory bowel diseases (Gijsbers, Van Assche et al. 2004), sepsis and acute respiratory distress syndrome (Cummings, Martin et al. 1999; Goodman, Cummings et al. 1999), while both it and the CXCR2 play roles in synovial

infiltration by neutrophils in arthritic joints (Podolin, Bolognese et al. 2002). However, numerous alternate GPCR are involved in neutrophilic inflammation, including those for LTB4, C5a, and fMLP, such that antagonism of either LTB4 or C5a has been shown to be of significant benefit in various inflammatory settings (Wollert, Menconi et al. 1993; Crooks, Bayley et al. 2000; Park, Tofukuji et al. 2000). The precise interrelationships of these mediators in inflammatory responses have not been formally determined, but it has been reported that signaling through the C5a or fMLP receptors can effectively desensitize the ELR-CXC chemokine receptors (Blackwood, Hartiala et al. 1996). On the other hand, CXCL8 reportedly poorly desensitizes some events (e.g., intracellular Ca^{2+} flux) associated with C5a and fMLP receptor signaling (Tomhave, Richardson et al. 1994; Richardson, Ali et al. 1995), although it can desensitize chemotactic responses driven by these ligands (Blackwood, Hartiala et al. 1996).

Some time ago we developed a very high affinity broad-spectrum ELR-CXC chemokine antagonist, bovine $CXCL8_{(3–74)}$K11R/G31P (bG31P) (Li, Zhang et al. 2002; Li, Zhang et al. 2002) based on a high affinity bovine CXCL8 analogue, $CXCL8_{(3–74)}$K11R (Li and Gordon 2001). This antagonist could block CXCL8 binding to neutrophils, as well as neutrophil activation by agonists present in wash fluids from the lungs of cattle with bacterial pneumonia. Bovine G31P fully antagonizes neutrophil responses to endotoxin challenge of cattle for more than two days after a single low-dose treatment (Li, Zhang et al. 2002), and blocks pyrexia, hemorrhagic consolidation, and pulmonary neutrophilia in airway endotoxemia in guinea pigs even if delivered after the onset of symptoms (Gordon, Li et al. 2005). We also subsequently developed numerous human-bovine chimeric forms of bovine G31P that were as effective as bG31P in blocking ELR-CXC chemokine-mediated pathology (Zhao, Li et al. 2007), and then developed a fully human form of G31P (hG31P). However, some of the protective effects of bovine G31P in airway endotoxemia (Gordon, Li et al. 2005), for example, seemed to be somewhat tangentially related to its putative neutrophil-centered effects. For example, G31P treatment reduces endogenous pyrogen expression and pyrexia prior to the time when neutrophils were appreciably present in the airways, suggesting that it may have effects on structural (e.g., epithelial) cells. Moreover, data from a study of its effects in aspiration pneumonia, wherein high level bacterial colonization of the lungs occurs, confirmed its effectiveness in

this environment as well (Chapter 5). In such cases the bacteria would release formyl peptides (e.g., fMLP) among other products and activate the complement cascade, leading to the generation of C5a. Thus, we wished to explore further the mechanisms by which G31P might interact with airway epithelial cells, but also its effect on neutrophil responses to ligands for heterologous GPCR.

4.2. MATERIALS AND METHODS

4.2.1. Reagents and supplies

The following reagents were purchased commercially: restriction endonucleases BamHI and EcoRI, T4 DNA ligase, DNA polymerase, fluo-4 AM, MEM [with L-glutamine], HEPES, lipofectamine 2000, MEM non-essential amino acid solution (Invitrogen Canada Inc, Burlington, ON); QIAprep Spin Miniprep, QIAquick Gel Extraction Kits, QIA shredder and RNeasy Mini Kit (QIAGEN Inc, Mississauga, ON); QuickChange™ Site-Directed Mutagenesis kit and Brilliant® qRT-PCR Master Mix Kit (Stratagene Cloning Systems, La Jolla, CA); glutathione-Sepharose, the expression vector pGEX-2T, benzamidine-Sepharose (Amersham-Pharmacia Biotech, Piscataway, NJ); aprotinin, dextran, endotoxin (*Escherichia coli* LPS, serotype 0127:B8), hexadecyltrimethylammonium bromide (HTAB), gelatin, isopropyl-thio-D-galactopyranoside (IPTG), luminol, phenol red-free Hank´s Balanced Salt Solution (HBSS), phenylmethysufonyl fluoride (PMSF), tetramethylbenzidine (TMB), Triton X-100, and dithiothreitol (DTT) (Sigma Chemical Co, Mississauga, ON); paired anti-CXCL8 capture and detection antibodies and recombinant CXCL8 (R & D Systems, Minneapolis, MN); rabbit anti-human lactoferrin antibody, recombinant lactoferrin and biotinylated anti-lactoferrin antibody (ICN Biomedicals, Inc, Irvine, CA); Lymphocyte Separation Medium density gradient medium (MP Biomedicals LLC, Aurora, ON); Diff-Quick stain kit (American Scientific Products, McGraw Park, IL); annexin V/PI staining kit (BD Biosciences, Rockville, MD); and five-week old female Hartley guinea pigs (Charles River Laboratories, Charles River, MA). All the experiments were carried out according to the guidelines established by the Canada Council on Animal Care and were approved by University of Saskatchewan animal ethics review panel.

4.2.2. Generation of hG31P

A full-length human CXCL8 cDNA was synthesized commercially (Takara Biotech, Dalian, China). From this we generated a glutathione-S-transferase (GST) fusion protein construct encoding amino acids 3-72 of human CXCL8, then introduced a Lys11-to-Arg substitution (we reported previously that bovine $CXCL8_{(3-72)}K11R$ is a very high affinity CXCR1/CXCR2 agonist; ref (Li and Gordon 2001). We next introduced a Gly31 to Pro substitution, to generate human $CXCL8_{(3-72)}$ K11R/G31P (hG31P). Each plasmid was transformed into competent HB101 cells and their sequences were verified commercially (Plant Biotechnology Institute, Saskatoon, SK). The methods for expressing the constructs, cleaving the target proteins from their GST fusion partners, and purifying these have been described in detail (Li, Zhang et al. 2002; Li, Zhang et al. 2002). Briefly, the recombinant bacteria were sonicated in the presence of the protease inhibitors aprotinin (2 mg/ml) and PMSF (50 mM), then the recombinant GST fusion proteins present in the sonicates were purified by affinity chromatography using glutathione-sepharose columns. The recombinant CXCL8 analogues were cleaved from their GST fusion partners by thrombin digestion and then further purified using benzamidine-sepharose columns. The relative molecular mass and purity of each protein was verified by SDS-PAGE and Western blotting with biotinylated anti-human CXCL8 antibody (Li and Gordon 2001; Li, Zhang et al. 2002).

4.2.3 Generation of CXCR1-transfected HEK293 cells.

HEK293 cells (CRL-1573; American Type Culture Collection [ATCC], Manassas, VA) were transfected with the gene for the CXCR1 in the vector pCDNA3.1+ (Missouri S&T cDNA resource center; www.cdna.org) using lipofectamine. The CXCR1-expressing cells were selected using gentamicin (800 μg/ml) and their expression of the CXCR1 gene was confirmed by FACS and qRT-PCR.

4.2.4. Neutrophil chemotaxis assay

Chemotaxis was assessed using modified Boyden chamber microchemotaxis assays (Li and Gordon 2001; Li, Zhang et al. 2002; Li, Zhang et al. 2002) with either purified neutrophils or CXCR1-transfected HEK cells. For the former, human peripheral blood leukocytes were fractioned on standard density gradients, and the neutrophils harvested from the bottom of the gradients and cleared of contaminating red blood cells by hypotonic lysis. The purified neutrophils (or HEK cells) were suspended at 2×10^6/ml in PBS^+ (phosphate-buffered saline [PBS; pH 7.4], 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 5 mM glucose, and 0.1% bovine serum albumin). The chemoattractants (e.g., CXCL8), either alone or together with hG31P, were placed in the lower compartment of the Boyden chamber wells and cells in the upper compartment, with the two compartments separated by polyvinylpyrrolidone-free, 5 (neutrophils) or 10 (CXCR1-HEK cells) μm pore-size polycarbonate filters. After incubation for 20 min (neutrophils) or 5 h (CXCR1-HEK cells) at 37°C in a 5% CO_2 atmosphere, the cells that had migrated into the filters were fixed and stained using a Diff-Quick kit. The numbers of cells responding in each well were enumerated by direct counting of at least five 40× objective fields, and the results expressed as the mean number of cells per 40x field ± SEM.

In a series of preliminary experiments we confirmed that CXCL1, CXCL5, and CXCL8 induced maximal neutrophil chemotactic responses at concentrations of 100, 100, and 10 ng/ml, respectively, while CXCL8 induced maximal CXCR1-HEK cell responses at 100 ng/ml. We also optimized the dilution of each sputum sample (i.e., 1:1 to 1:100) required to maximize its neutrophil chemotactic activity. We tested the effects on sputum-induced chemotactic responses by using hG31P at 0.1-100 ng/ml, and found that the antagonistic effects observed at 10 ng/ml were not altered by increasing the dose of hG31P (data not shown).

4.2.5. Reactive oxygen intermediate release assay

We used total white blood cell (WBC) preparations for the reactive oxygen intermediate (ROI) release assays. To generate WBC, heparin-anticoagulated human blood was mixed with an equal volume of 6% (w/v) dextran (75 kD) in PBS, and then the

tubes were allowed to stand upright and undisturbed for ≈1 h at room temperature. When the red blood cells had sedimented, the upper phase, comprising plasma, platelets and WBC, was centrifuged and the cell pellet resuspended at 2.5×10^6 cells/ml HBSS. For the assays, we mixed 50 µl of WBC, 25 µl of human CXCL8 (final concentration, 100 ng/ml) or HBSS, 25 µl of HBSS or the indicated concentrations of hG31P, and 50 µl of luminol (0.3 mM). We had confirmed in preliminary experiments that 100 ng/ml CXCL8 was optimal for induction of WBC ROI release, as determined by chemiluminescence using a microplate spectrofluorimeter (NovoStar, BMG LABTECH Inc. Durham, NC). The data are expressed as the maximum sample luminescence minus that of cells exposed to HBSS alone.

4.2.6. Assay of intracellular Ca^{2+} flux

Cells were purified as described above, and then washed twice with Ca^{2+}-free PBS^+ medium and suspended at 5×10^6 cells/ml in the same medium. The cells were stained for 30 min at 37°C with 2 µM fluo-4 AM, then washed twice with Ca^{2+}-free PBS^+, resuspended at 4×10^6 cells/ml in PBS^+ containing 3.3 nM Ca^{2+}, and kept at room temperature until used for testing. Prior to challenge, the cells were incubated for 15 min with medium or hG31P (100 ng/ml), then challenged with medium or human CXCL1, CXCL5, or CXCL8 (each, 100 ng/ml). In preliminary experiments we had confirmed that 100 ng/ml induced optimal intracellular Ca^{2+} flux in these cells, as determined using a microplate spectrofluorimeter (emission wavelength, 520 nm; excitation wavelength, 488 nm).

4.2.7 Heterologous desensitization of GPCR.

Neutrophils were purified as above and resuspended in PBS^+ medium at 2×10^6 cells/ml, then stimulated with 0.1 nM C5a, 1 nM fMLP, or 1 ng/ml LTB4 in the presence of 0, 10 or 50 ng/ml hG31P. The impact of the hG31P on the agonist-induced intracellular Ca^{2+} flux and chemotactic responses were measured as noted above. These experiments were repeated three times.

4.2.8. Sputum samples

Sputum samples were collected for diagnostic purposes from 12 cystic fibrosis or bronchiectasis patients with bacterial pneumonia. As controls for samples not associated with bacterial infections, sputa from two subjects with COPD or asthma were also obtained. For processing, the sputum mucus plugs were dispersed by treatment with 1% DTT/HBSS, then the cells were sedimented and the supernatants dialysed extensively against multiple changes of PBS, as noted previously (Swystun, Gordon et al. 2000; Gordon and Li 2002; Gordon, Swystun et al. 2003). The levels of CXCL8 in each sample were determined by ELISA, as noted (Gordon, Swystun et al. 2003). The supernatants were aliquoted and stored at -80˚C. Sputum from healthy donors did not induce any significant neutrophil chemotactic responses (data not shown).

4.2.9. Effects of hG31P on responses of A549 bronchial epithelial cells

Human A549 bronchial epithelial cells (ATCC No.CCL185) were grown to a maximum of 90% confluence in 24-well plates in MEM (with L-glutamine) supplemented with 10% FBS, 0.01 M HEPES, and non-essential amino acids. Prior to stimulation, the cell monolayers were washed and provided with fresh medium, then exposed for 15 min at room temperature to 1-100 ng/ml of hG31P. The indicated doses of LPS or $0 – 1.28\times10^{6}$ unstimulated purified neutrophils from healthy donors were added to the cells, which were incubated for 16 h at 37 °C before the supernatants were collected. Total RNA was collected from the cells using commercial extraction kits and standard approaches. ELISA assays were used to quantify cytokine levels in the supernatant fluids, while qRT-PCR was used to quantify the relative mRNA levels.

4.2.10. ELISA assay of lactoferrin and chemokines

Our ELISA protocols have been reported in detail previously (Gordon, Swystun et al. 2003). Culture supernatants were not diluted before the assays, while BAL fluids for lactoferrin assays were diluted 1:10. The data are presented as pg/ml (IL-8) or ng/ml (lactoferrin), based on recombinant protein standard curves. The cytokine ELISAs were

sensitive to 5–10 pg/ml, while the lactoferrin assay was sensitive to 5 ng/ml. Our CXCL8 ELISA does not detect hG31P, as the capture antibody does not recognize this molecule (JRG, unpublished observation).

4.2.11. Quantitative real time PCR (qRT-PCR)

Total cellular RNA was quantified spectrophotometrically and stored at -80°C. For the qRT-PCR, we used a commercial one-step master mix kit, with the thermal profile programmed as: first segment, 50°C for 30 min, and 95°C for 10 min (1 cycle); second segment, 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec (40 cycles); and third segment, the reading was taken at 72°C during the 30-sec plateau. The sequences of the qRT-PCR primers were: GAPDH forward, 5'- TGC CTC CTG CAC CAC CAA C-3' and reverse, 5'- GGC CAT CCA CAG TCT TCT GG-3'; CXCL1 forward, 5'- CCC AAG AAC ATC CAA AGT GTG A-3' and CXCL1 reverse, 5'- GTC ACT GTT CAG CAT CTT TTC G -3'.

4.2.12. Neutrophil apoptosis

Human neutrophils were isolated as described above and resuspended at 5×10^6 cells/ml in PBS^+, then aliquoted into 96-well plates at 100 µl per well. Medium (PBS^+) alone or containing hG31P (100 ng/ml) was added to the cells and incubated for 15 min at room temperature, before addition of CXCL1 or CXCL8 (100 ng/ml) or carrier medium alone. After 24 h at 37°C the cells were stained as per the manufacturer's instructions using FITC-labeled annexin V and propidium iodide for FACS detection of apoptotic and necrotic cells, respectively, then analyzed within one hour using FACScan flow cytometer.

4.2.13. *In vivo* confirmation of the anti-inflammatory activities of G31P

The assessment of hG31P's effect on 15 h airway endotoxemia pathology in guinea pigs has been described previously for the bovine and human-bovine chimeric forms of G31P (Gordon, Li et al. 2005; Zhao, Li et al. 2007). Briefly, for challenge the animals (n = 5) were challenged with a 200 µl bolus of LPS (5 µg/kg) in sterile saline by intranasal

intubation. At 30 min before challenge, 250 µg/kg hG31P or an equivalent volume (1 ml) of saline was given s.c. and at 4, 8, or 16 h post-challenge the animals were euthanized with halothane. Bronchoalveolar lavage fluid (BAL) was collected using 2.0 ml volumes of sterile saline. BAL total WBC and red blood cells were enumerated by direct counting, and the results expressed as the mean number of cells per BAL sample (± SEM). BAL cell differentials were determined using Wright's solution-stained BAL cell cytospin preparations and the total numbers of neutrophils were calculated using these data. All the data are expressed as means ± SEM. All BAL fluids were assessed independently (either immediately or after storage at –80°C) for myloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9), MMP-2, and lactoferrin (LF). This experiment as repeated twice.

4.2.14. Myeloperoxidase assay

BAL fluids (10 µl) were diluted 10-fold with 0.5% HTAB and incubated at room temperature for 10 min with 10 volumes of TMB, before stopping the reactions with 10 volumes of 1M phosphoric acid. The reactions were quantified by spectroscopy (OD, 450 nm) and the data expressed as the mean OD_{450} ± SEM. Each sample was assayed in triplicate.

4.2.15. Assessment of BAL fluid MMP-9 levels

BALF levels of MMP-2 and -9 were measured by gel zymography, as previously described (Chakrabarti and Patel 2005). Briefly, 20 µl samples of BAL sample were run on 7.5% SDS-PAGE gels containing 1 mg/ml gelatin. The gels were incubated overnight at room temperature in 2.5% Triton X-100, 50 mM Tris-HCl, 10 mM calcium chloride, 1 mM zinc chloride, rinsed in deionized water for 30 min, and then incubated overnight at 37°C in 50 mM Tris-HCl, 10 mM calcium chloride, 1 mM zinc chloride, before staining with Coomassie brilliant blue. The identities of the gelatinolytic MMP-2 and -9 bands were determined based on their molecular weights and comparison with standards prepared from homogenates of untreated HT1080 fibrosarcoma cells (ATCC CCL21)(Sariahmetoglu,

Crawford et al. 2007). The results are expressed as relative densitometry units for each gelatinase band.

4.2.16. Statistical analysis

Two-group comparisons were made using student's t-tests (two-tailed). We used Spearman Rank correlation analyses to assess the correlations between the sputum cytokine levels and their associated responses. All results are expressed as the mean ± SEM.

4.3. RESULTS

4.3.1. Human CXCL8$_{(3-72)}$K11R/G31P effectively antagonizes both CXCR1- and CXCR2-dependent activation of neutrophils.

To confirm unequivocally whether human CXCL8$_{(3-72)}$K11R/G31P (hG31P) acts at least in part through the CXCR1 we employed CXCR1-transfected HEK293 cells. We confirmed by FACS (data not shown) and qRT-PCR (Fig. 4.1A) that these cells express this receptor but not the CXCR2 (data not shown) and that 100 ng/ml CXCL8 induced chemotactic and intracellular Ca^{2+} flux responses. At 10 ng/ml hG31P blocked CXCL8-induced chemotactic responses by ≈80%, while at 100 ng/ml (but not 10 ng/ml; data not shown) it dramatically reduced intracellular Ca^{2+} flux (Fig. 4.1A). Given that the biological differences between CXCR1-transfected HEK cells and neutrophils, we also confirmed with freshly purified human neutrophils that hG31P blocked CXCL8 (10 ng/ml)-induced chemotaxis and reactive oxygen intermediate (ROI) release (Fig. 4.1B). It is noteworthy that neutrophil superoxide release is reportedly attributable exclusively to CXCR1 signaling (15). We also found that 10 ng/ml hG31P markedly attenuated intracellular Ca^{2+} flux induced by 100 ng/ml CXCL8 (Fig. 4.1B). We then assessed hG31P's ability to block activation of neutrophils induced by CXCR2-exclusive ligands. As with CXCL8, hG31P dose-dependently blocked chemotactic responses of neutrophils to CXCL1 and CXCL5 (both 100 ng/ml), while 10 ng/ml hG31P ≥95% reduced intracellular Ca^{2+} flux induced by an optimal dose (100 ng/ml) of either ligand (Fig.4.1C). It is noteworthy that, overall, CXCL8 was a

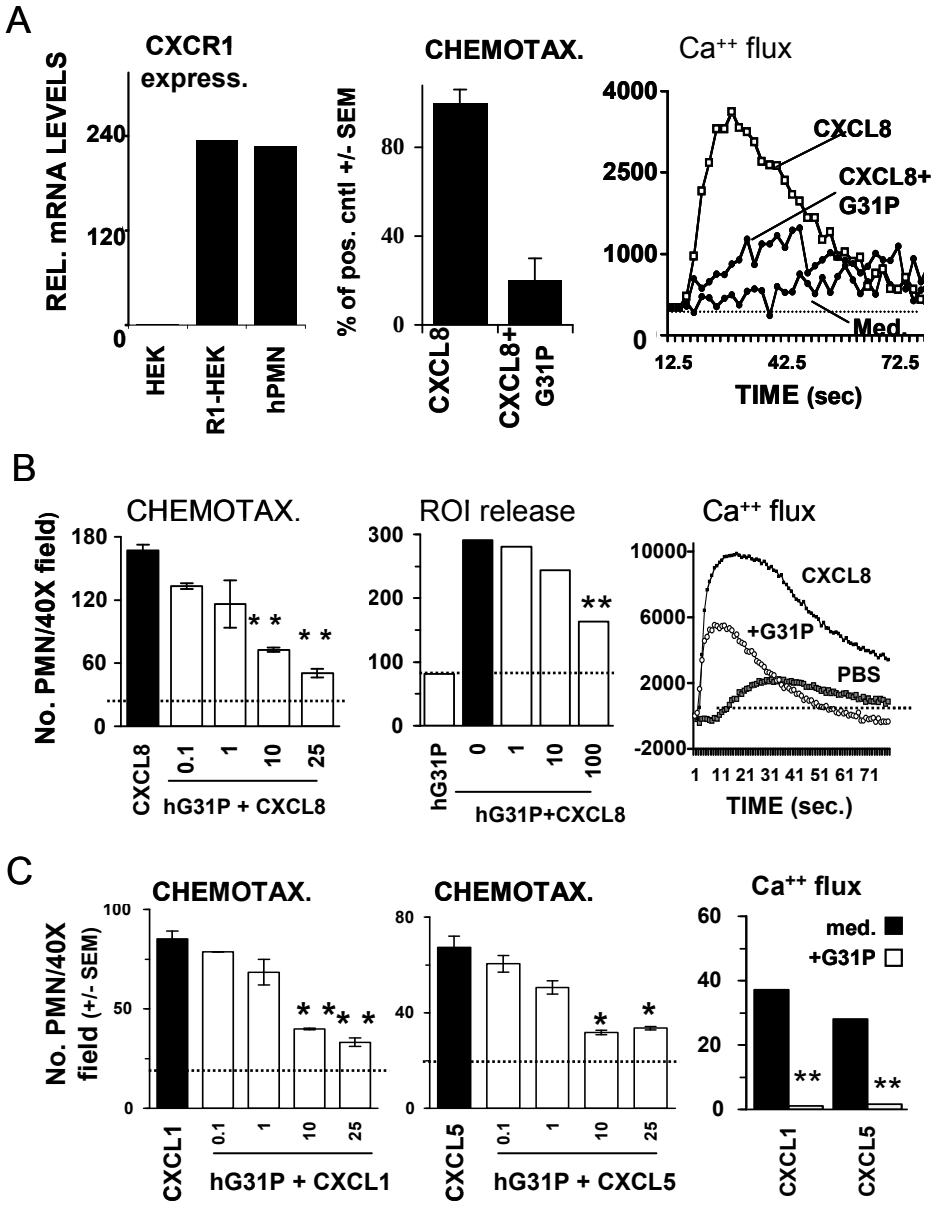
substantially stronger agonist than either CXCL1 or CXCL5 in terms of inducing this intracellular Ca^{2+} response.

4.3.2. Human G31P induces heterologous desensitization of alternate G protein-coupled receptors.

Numerous neutrophil agonists, including C5a, LTB4, ELR-CXC chemokines, and fMLP, signal into these cells via distinct G protein-coupled receptors (GPCR). It has been shown previously that signaling through C5a, fMLP, or CXCL8 can reciprocally desensitize neutrophil chemotactic responses to subsequently introduced heterologous ligands (Blackwood, Hartiala et al. 1996). On the other hand, CXCL8 reportedly does not effectively desensitize C5a- or fMLP-induced neutrophil activation as assessed by intracellular Ca^{2+} flux (Tomhave, Richardson et al. 1994; Richardson, Ali et al. 1995). In assessing G31P's relative capacity to affect heterologous GPCR signaling, it is important to recognize that G31P has a much higher affinity for its receptors than do the ELR-CXC chemokines. Thus, bovine G31P inhibits by 50% the chemotactic responses of neutrophils to a ≈250-fold higher concentration of native ligand (i.e., CXCL8) (Li, Zhang et al. 2002). We wished to assess then the extent to which hG31P would desensitize neutrophil responses to ligands such as C5a, fMLP, and LTB4, each of which are relevant neutrophil agonists in inflammatory conditions. Thus we examined hG31P's impact on intracellular Ca^{2+} flux and chemotactic responses induced by each of these (Fig.4.2.). We found that 0.1 nM C5a induced marked Ca^{2+} flux in neutrophils, as well as chemotactic responses, and that the addition of hG31P reduced C5a-mediated chemotaxis by ≥50%, although its impact on intracellular Ca^{2+} flux was substantially more modest. The bacterial tripeptide fMLP induced a much more robust Ca^{2+} flux response than did C5a, but an essentially equivalent chemotactic response. The hG31P treatment very substantially reduced both the Ca^{2+} and chemotactic (85% reduced) responses. LTB4 was a strong neutrophil agonist in both assays and, as with fMLP, hG31P substantially reduced LTB4-induced Ca^{2+} flux and chemotactic (≈67% reduction) responses. Taken together, these data show that hG31P did dampen neutrophil responses to each of these heterologous GCPR ligands.

Figure 4.1. The ELR-CXC chemokine antagonist CXCL8$_{(3-72)}$K11R/G31P operates by targeting the CXCR1 and CXCR2 on neutrophils.

(A) We generated CXCR1-transfected HEK293 cells and used these to determine whether human (h) G31P acts via the CXCR1. We used qRT-PCR to assess CXCR1 expression by control and CXCR1-transfected HEK cells and compared this expression with that of peripheral blood neutrophils (left panel). For the chemotaxis assays, responses to an optimized concentration of CXCL8 (10 ng/ml) were assessed using modified Boyden chamber microchemotaxis assays, as noted in the *Materials and Methods*. The ability of hG31P (10 ng/ml) to antagonize this response was assessed by simultaneously exposing the cells to the agonist and antagonist. The results are expressed as the mean (±SEM) number of cells/40x objective microscope field (middle panel). For the Ca^{2+} flux assays, neutrophils were labeled with the Ca^{2+} indicator dye fluo-4AM and assayed in the presence (100 ng/ml) or absence of hG31P for chemokine (100 ng/ml) - induced intracellular Ca^{2+} flux as described in the *Materials and Methods* (right panel). **(B)** We also assessed whether hG31P could antagonize CXCL8-dependent chemotaxis, reactive oxygen intermediate (ROI) release, and intracellular Ca^{2+} flux responses by purified human neutrophils using the same approaches as in panel A. **(C)** In order to confirm that hG31P also acted via the CXCR2 we tested its ability to antagonize neutrophil chemotactic and intracellular Ca^{2+} flux responses induced by the CXCR2-exclusive ligands CXCL1 and CXCL5 (both 100 ng/ml). Our data confirm that hG31P did effectively antagonize both CXCR1- and CXCR2-dependent responses. * and **, $p < 0.05$ and 0.01, respectively, relative to the chemokine treatments alone. The data shown are representative of at least three experiments performed with similar results. The dashed lines across graphs in panels A, B, and C represent the mean background response in the indicated assays.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CHEMOTAX.
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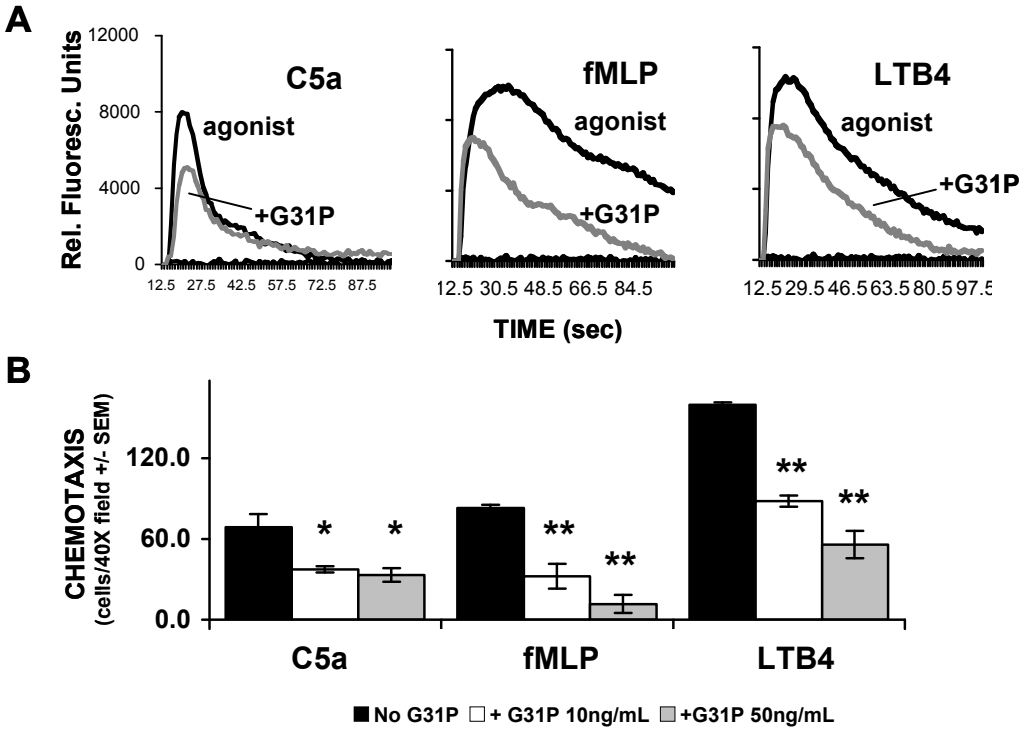


Figure 4.2. Human G31P engagement of neutrophils induces heterologous desensitization of their G protein-coupled receptors for C5a, LTB4, and fMLP.

Purified Purified human neutrophils were stimulated with 0.1 nM C5a, 1 nM fMLP, or 1 ng/ml LTB4, in the absence or presence of 10 or 50 ng/ml of hG31P, as described in the *Material and Methods*. (A) The ability of hG31P (10 ng/ml) to interfere with the agonist-induced intracellular Ca^{2+} flux was assessed as in Fig. 1. (B) The abilities of varying doses of hG31P to block the cells' chemotactic responses were also assessed as above. G31P very substantially reduced Ca^{2+} flux induced by fMLP and LTB4, but was less effective in blocking C5a-dependent Ca^{2+} flux, and this relative efficacy was observed also in the chemotaxis assays. * and **, $p < 0.05$ and 0.01, respectively, relative to the agonist treatments alone. The data shown are representative of at least three experiments performed with similar results. The dashed lines across graph in panel B represents the mean background response in the assay.

4.3.3. Human G31P effectively antagonizes the inflammatory mediators present in sputum from bacterial pneumonia patients

As suggested above, inflammatory diseases are marked by the simultaneous expression of numerous neutrophil agonists. Sputum from cystic fibrosis (CF) patients, for example, can contain CXCL8 and other CXC chemokines, LTB4, formyl peptides (e.g.,, fMLP), and perhaps C5a (Mackerness, Jenkins et al. 2008). Thus, given that our data documenting that hG31P can variably desensitize these receptors we wished to know just how effective it would be in blocking the agonists present in inflammatory samples. Thus, we obtained a bank of sputa from 12 CF and bronchiectasis patients being treated for bacterial pneumonia and directly tested the impact of hG31P on their neutrophil chemotactic activities (Fig. 4.2.). The disease status of the CF donors was classified as advanced, severe, moderate or mild, and that of the bronchiectasis donors as severe or moderate, or frank bacterial pneumonia, based on clinical criteria. We included sputum from one asthmatic and one chronic obstructive pulmonary disease (COPD) subject as controls. We assessed the levels of CXCL8 in each sputum sample (Fig. 4.3.; values above the sample bars) and found that these correlated positively with the samples' ability to induce neutrophil chemotaxis ($r=0.63$ [14 DF], $p=0.013$). At 10 ng/ml hG31P ≈60-95% inhibited the chemotactic responses of neutrophils from healthy blood donors to the sputum from 10 of the 12 cystic fibrosis and bronchiectasis donors. Only sputum from the two donors classified as having advanced cystic fibrosis (donors 1 and 2) appeared more or less resistant to the antagonistic effects of hG31P in this assay. Interestingly, the relative ability of hG31P to block sputum-induced neutrophil chemotactic activities (i.e., percent inhibition) was not significantly correlated with the levels of CXCL8 in the samples ($r=0.369$ [14 DF], $p=0.15$). For example, sample 1 contained 3.09 ng/ml of CXCL8 and was only modestly affected by hG31P, while sample 10 contained 3.32 ng/ml CXCL8 and its activity was almost ablated by the hG31P treatment. The sputum from the asthmatic and COPD subjects also induced marked neutrophil responses and hG31P largely blocked these too; we have reported previously that sputum from asthmatic subjects contains high levels of CXCL8 (Gordon and Li 2002).

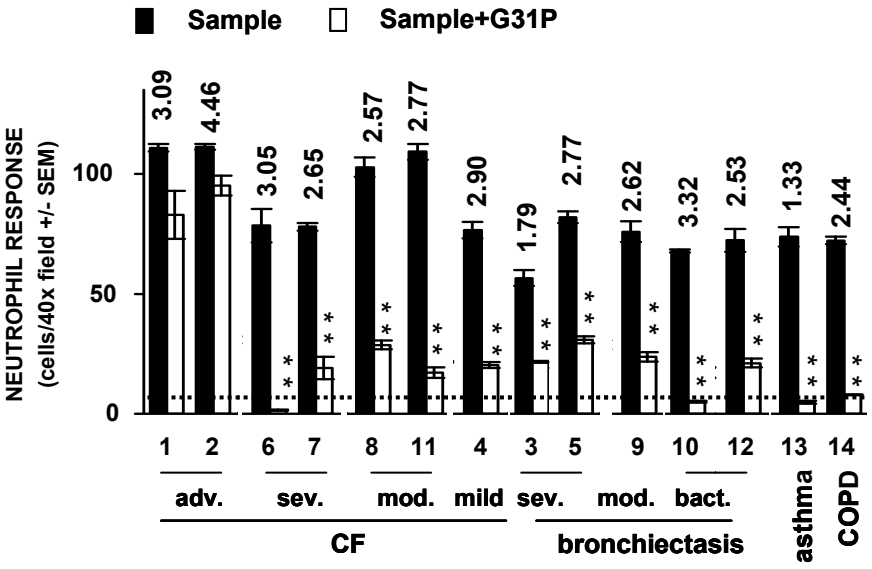


Figure 4.3. Human G31P antagonizes the neutrophil chemotactic activities present in sputum from cystic fibrosis (CF) and bronchiectasis patients with bacterial pneumonia.

Sputum samples were collected from 12 CF or bronchiectasis patients with bacterial pneumonia as well as two control subjects with COPD or asthma, and assayed for their CXCL8 content (values above the black bars). The abilities of hG31P (10 ng/ml) to antagonize chemotactic responses of neutrophils from healthy donors to optimized dilutions of the sputa were assessed as in Fig. 4.1. The data are expressed as the mean (±SEM) number of cells/40x objective microscope field. The assays were repeated twice and gave essentially the same results each time. Sputum from healthy donors did not induce any significant neutrophil chemotactic responses (data not shown). With the exception of samples 1 and 2, G31P significantly reduced the cells chemotactic responses. **, $p \leq 0.01$ versus no G31P treatment. The chemotaxis assays were repeated twice. The dashed line across the graph represents the mean background response in the assay.

4.3.4. Human G31P blocks ELR-CXC chemokine-mediated anti-apoptotic effects in neutrophils.

As neutrophils leave the vasculature or become effete they rapidly undergo programmed cell death or apoptosis as a means of preventing adventitious host pathology. However, during inflammatory events it is in the best interest of the host to maintain these cells in a viable state such that they can effectively engage microbial targets. An array of inflammatory mediators, including the ELR-CXC chemokines, have anti-apoptotic effects on neutrophils (Glynn, Henney et al. 2002). We wished to know then whether hG31P's anti-inflammatory effects would extend to reversing chemokine-induced anti-apoptotic processes, thereby allowing neutrophils to quickly progress into apoptosis. We used FITC-annexin V binding as our assay of apoptosis. We stained freshly purified neutrophils with this marker as a negative control (shaded signal, Fig. 4.4.) and cells that had been cultured for 18 h in medium alone as an apoptosis positive control (Fig. 4.4., upper panel). The freshly purified cells appeared to have two levels of annexin binding, with most cells being more or less negative and a smaller proportion binding moderate levels of this apoptosis marker. When we added CXCL8 to the cells (Fig. 4.4., middle panel, dashed line) there was a marked diminution of the numbers of cells that strongly bound annexin V and the addition of hG31P to these cultures fully reversed this CXCL8-induced anti-apoptotic activity. CXCL1 also had protective effects on neutrophils, but they were subtly different than those of CXCL8 (Fig. 4.4, bottom panel, dashed line). The CXCL8-protected cells had largely bound intermediate levels of annexin V, while those incubated with CXCL1 bound either very low or intermediate levels of annexin. The addition of hG31P to the CXCL1 cultures induced a substantial proportion of the cells to upregulate their apoptotic processes, but nevertheless there remained a significant population of cells that bound intermediate levels of annexin.

4.3.5. Human G31P antagonizes the inflammatory cycle in endotoxin-challenged bronchial epithelial cells

Exposure of the airway epithelium to bacterial endotoxin triggers a pulmonary inflammatory response, wherein these cells secrete CXCL8, for example, which directly fosters neutrophil recruitment into this tissue compartment. While we have shown that

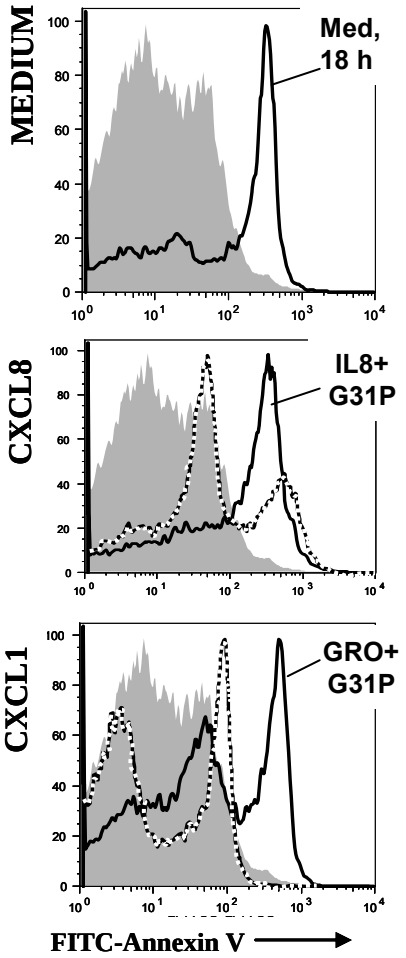


Figure 4.4. Human G31P reverses the anti-apoptotic influence of CXCL1 and CXCL8 on neutrophils.

Purified human neutrophils were cultured for 18 h in medium alone (apoptosis positive control; top panel) or in the presence of 100 ng/ml CXCL1 or CXCL8, either with (solid lines) or without (dashed lines) hG31P (100 ng/ml), and stained with fluorochrome-labeled annexin V. The proportions of apoptotic (annexin V-binding) cells were determined by FACS. Negative control results obtained using freshly purified annexin V-stained neutrophils (shaded tracing) are included in each graph. Necrotic cell death was also assessed by propidium iodide uptake; we found ≤3% propidium iodide-positive cells in all samples. The data indicate that cells incubated in medium alone were largely apoptotic. Exposure to CXCL8 or CXCL1 reduced apoptosis very substantially, but addition of hG31P to these cultures largely reversed the ELR-CXC chemokine-induced anti-apoptotic effects. These assays were repeated three times.

hG31P antagonizes the impact of the ELR-CXC chemokines on neutrophil responses, little attention is paid to whether epithelial cells themselves respond to the ELR-CXC chemokines they elaborate in airway endotoxemia, or to the neutrophils they recruit. We thus decided to investigate this using human type II alveolar A549 epithelial cells that we challenged with bacterial endotoxin (Fig 4.5.) in the presence or absence of hG31P. The quiescent A549 cells secreted very little CXCL8, but LPS dose-dependently up-regulated their expression of this chemokine. Reports of CXCR1 and CXCR2 expression by epithelial cells vary, so we wished first to determine whether our A549 cells expressed either of these receptors. We used qRT-PCR and did indeed detect significant levels of the CXCR1 (Fig. 4.5A, right panel) but little in the way of CXCR2. When we challenged our A549 cells with 10 ng/ml LPS in the presence of increasing doses of hG31P, the cells produced progressively less CXCL8 (Fig. 4.5B). This effect extended also to their expression of CXCL1, wherein hG31P reduced LPS-induced CXCL1 expression by ≈98%, as determined by qRT-PCR.

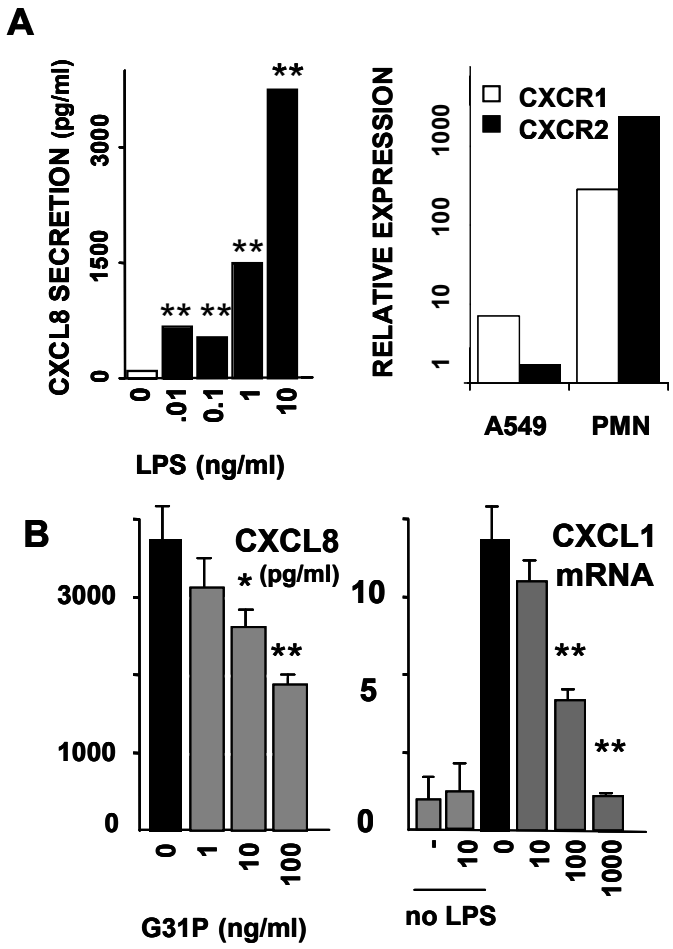
Neutrophils release a variety of mediators that are potential epithelial cell agonists (e.g., elastase; (Chen, Lin et al. 2004), while epithelial cells similarly secrete neutrophil agonists. This suggests the possibility that these cells could through a putative mutual stimulatory mechanism drive an escalating inflammatory process. We tested this by co-culturing purified otherwise quiescent neutrophils with monolayers of resting A549 cells. While the neutrophils were largely unstimulated, there was a significant background release of CXCL8 by these cells over 16 h in culture (Fig. 4.6). Nevertheless, simple co-culture of these neutrophils with A549 cells induced a synergistic expression of CXCL8 in the cultures, and this effect was dependent on the numbers of neutrophils added to the cultures (Fig. 4.6). The A549 cells did not secrete significant amounts of CXCL8 when cultured on their own. The addition of hG31P to these co-cultures dramatically (up to 98%) reduced the expression of CXCL8 ($p \leq 0.01$; Fig. 4.6.).

4.3.6. Human G31P blocks airway endotoxemia-induced acute lung inflammation

The observations that a fully human form of G31P blocked inflammatory responses at multiple levels (e.g., heterologous desensitization of GCPR, pro-apoptotic effects, blockade of the autocrine epithelial inflammatory cycle) suggested that it could be effective

Figure 4.5. Human G31P antagonizes the endotoxin-induced inflammatory cycle in human bronchial epithelial cells.

Monolayers of A549 bronchial epithelial cells were exposed to (**A**) varying doses of endotoxin, and the culture supernatant collected 16 h later and assayed by ELISA for CXCL8 (left panel). Expression of the CXCR1 or CXCR2 by the A549 cells and human neutrophils was assessed by qRT-PCR using specific primers (right panel). Our data indicate that peak CXCL8 expression was found in the A549 cultures that were challenged with 10 μg/ml LPS, and that our A549 cells expressed significant levels of CXCR1, though certainly much less than that found in neutrophils. (**B**) A549 cell monolayers were challenged with 10 μg/ml of LPS in the presence of the indicated concentrations of hG31P, and 16 h later the culture supernatants and cellular RNA was isolated and assayed for CXCL8 protein (ELISA) and CXCL1 mRNA (qRT-PCR). Addition of hG31P to the A549 cells dose-dependently suppressed expression of both chemokines by the LPS-challenged cells. * and **, $p < 0.05$ and 0.01, respectively, relative to the baseline treatments alone. The data shown are from one experiment that is representative of at least three experiments performed.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CXCL8 SECRETION (pg/ml)
3000
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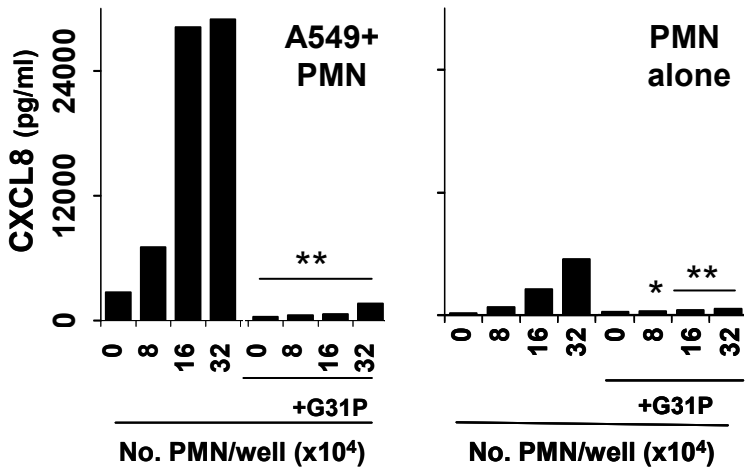


Figure 4.6. Co-culture of quiescent A549 cells with unstimulated peripheral blood neutrophils leads to syngergistic CXCL8 expression.

The indicated numbers of quiescent, purified human peripheral blood neutrophils from healthy donors were either cultured alone (PMN alone) or added to monolayers of unstimulated A549 cells (A549+PMN). G31P (100 ng/ml) was added to half of these cultures and 16 h later the culture supernatants were harvested assayed for CXCL8 by ELISA. The addition of neutrophils to the A549 cells synergistically enhanced expression of CXCL8 in a cell concentration-dependent fashion, and hG31P antagonized this cytokine response. * and **, $p < 0.05$ and 0.01, respectively, relative to the no hG31P treatments alone. The data shown are from one blood donor that is representative of the responses of three such donors.

also in blocking inflammatory responses *in vivo*. We have shown that bovine and bovine-human chimeric orthologues of human G31P can block an array of inflammatory pathologies, including airway endotoxemia (Gordon, Li et al. 2005; Zhao, Li et al. 2007), aspiration pneumonia (footnote 3), ischemia-reperfusion injury (Zhao et al, manuscript in preparation), and environmental pollutant-induced lung injury (Podechard, Lecureur et al. 2008). While we know in airway endotoxemia that bovine G31P treatments reduce neutrophil infiltration of the airways, it remains possible that neutrophils marginate in the pulmonary vasculature under the influence of the inflammatory mediators expressed in this compartment and are activated *in situ*. For this reason we wished to determine whether we could detect markers of the individual neutrophil granules or of activated structural cells in the lungs of endotoxemic animals. We induced airway endotoxemia in guinea pigs as reported previously (Gordon, Li et al. 2005; Zhao, Li et al. 2007) and assessed the levels of myeloperoxidase (MPO; 1˚ granules), lactoferrin (LF; 2˚ granules), and matrix metalloproteinase-9 (MMP-9; 3˚ granules) at 4, 8, and 16 h after endotoxin challenge. We confirmed that hG31P did indeed reduce airway neutrophilia (90-99% reduced at 4, 8 and 16 h; $p \leq 0.01$ versus saline-treated animals). In concert with this there were significant reductions in the BAL levels of myeloperoxidase (MPO; $p \leq 0.05$, 8 h time-point only), lactoferrin (LF; $p \leq 0.05$, all times), and matrix metalloproteinase-9 (MMP-9; $p \leq 0.05$, 4 and 8 h time-points), which suggested that few neutrophils that did achieve the lungs were not sufficiently activated to degranulate. We coincidentally observed a strong signal from a 68 kD gelatinase (Fig. 4.7.) that co-migrated with MMP-2 expressed in positive control HT1080 fibrosarcoma cells (data not shown), suggesting that we had indeed strongly activated the lung epithelium and other structural cells that express this metalloproteinase (D'Ortho, Jarreau et al. 1994; Fligiel, Standiford et al. 2006). G31P treatment also essentially abrogated this response at each time suggesting that, as we had observed with our A549 cells, structural cells were also influenced by ELR-CXC chemokine antagonism *in vivo*.

4.4. DISCUSSION

We have generated a human orthologue of bovine $CXCL8_{(3–74)}$K11R/G31P (bG31P; ref.(Li and Gordon 2002; Li, Zhang et al. 2002). We show in this study that this human

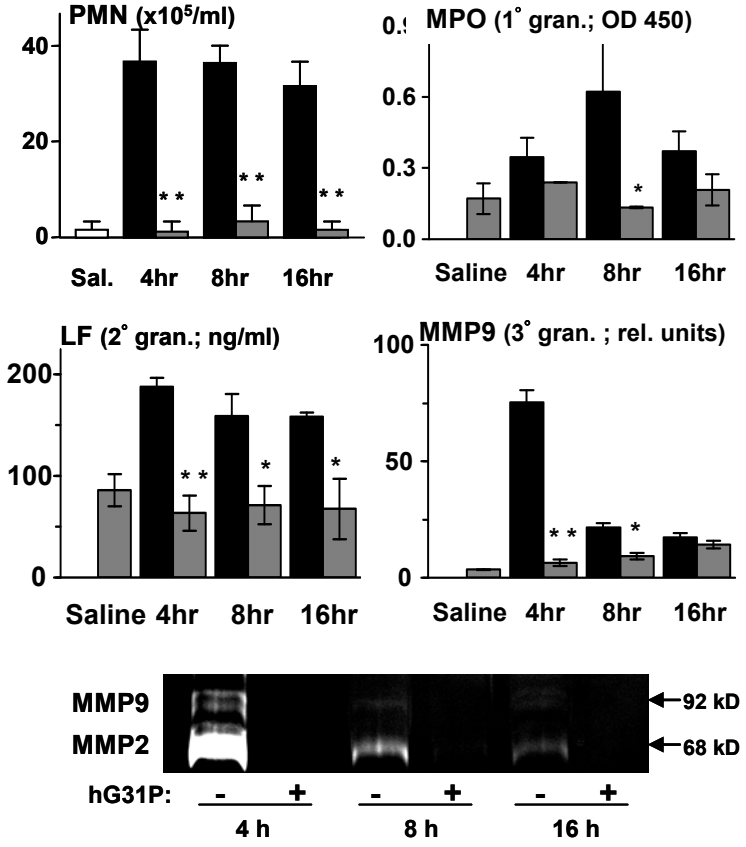


Figure 4.7. Human G31P treatments of airway endotoxemic animals reduce neutrophil recruitment and activation within the airways.

Airway endotoxemia was induced in guinea pigs by i.n. instillation of bacterial endotoxin (5μg/kg). The animals (n=5) were treated 30 min prior to challenge with either saline (black bars) or G31P (grey bars; 250 μg/kg) and euthanized 4, 8, or 16 h later. Differential counts were done on the cells recovered by bronchoalveolar lavage (BAL) and the BAL fluids were assessed for the levels of myeloperoxidase (MPO), lactoferrin (LF), and matrix metalloproteinase-9 (MMP-9), as specific markers of neutrophil primary, secondary, and tertiary granules, respectively, as noted in the materials and methods. The MMP-9 graphical data are drawn from the zymogram (lower panel) by gel scanning densitometry. The zymogram shows the BAL levels of MMP-9 (92 kD) and MMP-2 (68 kD), an activation marker of lung structural cells. The G31P treatments dramatically reduced neutrophil activation in the airways as well as structural cell activation. *, $p<0.05$ and **, $p<0.01$ as compared with LPS plus saline treatment only. Data are expressed as the mean ± SEM, and are representative of two repeat experiments.

G31P specifically blocks activation of neutrophils through the CXCR1 and CXCR2, and thereby their chemoattraction into inflammatory foci and expression of reactive oxygen intermediates (ROI). In engineering bovine G31P from its parent molecule, CXCL8, our first step was to introduce high affinity into the molecule, which we did by amino terminal truncation and substitution of a lysine in place of Arg11 to generate CXCL8$_{(3-74)}$K11R (Li and Gordon 2001). These arguments were in part based on prior work with human CXCL8 by Ian Clark-Lewis (Clark-Lewis, Schumacher et al. 1991; Clark-Lewis, Dewald et al. 1994), although the bovine form of CXCL8$_{(3-74)}$K11R appeared to have unexpectedly high agonist activity relative to its closest human counterpart (Li and Gordon 2001). We next reduced the receptor signaling activity of this agonist by introducing a Gly residue in place of Pro31 to generate an antagonist that fortuitously had exceptionally high affinity relative to CXCL8 (Li, Zhang et al. 2002). We hypothesize that it is this augmented receptor affinity that allows hG31P to so effectively compete with native ELR-CXC chemokines for their receptors on neutrophils. It makes sense that G31P would operate via both the CXCR1 and CXCR2 because its parent molecule was CXCL8, a high affinity ligand of both receptors (Jones, Wolf et al. 1996; Jones, Dewald et al. 1997). Our data showing that hG31P blocks CXCL8-dependent intracellular Ca^{2+} flux and chemotaxis in CXCR1-transfected HEK cells confirms unequivocally that hG31P targets the CXCR1, and the fact that it blocks the activities of CXCL1 and CXCL5, which are exclusive CXCR2 agonists (Jones, Wolf et al. 1996; Jones, Dewald et al. 1997), similarly confirms its interactions with these receptors.

We also documented herein that hG31P desensitizes heterologous GPCR in a quantitatively better fashion than CXCL8 (Blackwood, Hartiala et al. 1996), likely in part because of its high affinity interactions with the CXCR1 and CXCR2 (Li, Zhang et al. 2002). Thus, hG31P blocked both intracellular Ca^{2+} flux and chemotactic responses of neutrophils to signaling via CD88/C5aR (the C5a receptor), BLT1 (the LTB4 receptor), and FPR (the fMLP receptor), although it was not as effective in desensitizing C5a-dependent Ca^{2+} flux as that associated with LTB4 or fMLP signaling. It has been reported that in responding to signals generated in inflammatory settings, neutrophils adopt a hierarchical response pattern, with responsiveness to agonists immediately responsible for recruitment from the vasculature (e.g., CXCL8, LTB4) being superceded as the cells

traverse the blood vessel wall by responsiveness to those that would draw the cell to the pathogen itself (e.g., C5a, fMLP) (Heit, Tavener et al. 2002). This effect is reportedly brought about by C5a- and fMLP-driven activation of MAPK in neutrophils, which dampens Akt activation, a critical event in responses to CXCL8 and LTB4 (Heit, Tavener et al. 2002). In the context of a bacterial infection, for example, this process would make a great deal of sense, since as the neutrophil moves into the tissues and towards the pathogen it must necessarily concentrate its resources on that target. In support of this, it had also been reported that while the fMLP and C5a receptors can cross-desensitize one another and the CXCL8 receptor, CXCL8 signaling cannot reciprocally desensitize Ca^{2+} flux or a number of other intracellular events downstream of the fMLP or C5a receptors (Tomhave, Richardson et al. 1994; Richardson, Ali et al. 1995). At odds with this, however, is the report that while the CXCR2 cannot desensitize the CD88 or FPR, CXCR1 signaling can do so, albeit at significantly lower levels that that seen with C5a or fMLP-induced CXCL8 receptor desensitization (Richardson, Pridgen et al. 1998). Also at odds is the report that CXCL8 signaling desensitizes neutrophil chemotactic responses to subsequent C5a or fMLP exposures (Blackwood, Hartiala et al. 1996). Our own data clearly indicated that hG31P desensitizes neutrophil responses to fMLP, C5a, and LTB4, as noted likely related to high affinity interactions with its receptors. This could in part explain its effectiveness in dampening the pathology associated with airway endotoxemia in our model. It is known that endotoxin activates C5 to generate C5a (Smedegard, Cui et al. 1989), and it seems reasonable to suggest that commercial endotoxin preparations such as we used would likely be contaminated with bacterial tripeptides (e.g., fMLP). Furthermore, given that LTB4 antagonism is of significant benefit in airway endotoxemia (Fink, O'Sullivan et al. 1993; Wollert, Menconi et al. 1993), it is also reasonable to suggest that this agonist would also have been present in our endotoxemic animals, although we did not assess this. This suggests that the neutrophilic pathology we observed could well be related to the combined effects of multiple agonists, including ELR-CXC chemokines, C5a, LTB4, and perhaps fMLP. We hypothesize that hG31P's ability to ameliorate this pathology is attributable to its ability to not only block the CXCR1 and CXCR2, but also desensitize these other GPCR.

Another important finding in this study was that hG31P also antagonized the responses of airway epithelial cells to bacterial endotoxin. Our data shows that A549 cells express the CXCR1, although epithelial expression of the CXCR1 or CXCR2 is not uniform. While unstimulated BEAS2B or colonic epithelial cells don't express the CXCR-1 or -2 (Dwinell, Eckmann et al. 1999; Farkas, Hahn et al. 2005), epithelial cell lines from cystic fibrosis patients (IB3-1 and CFBE41o-) do express these receptors (Boncoeur, Criq et al. 2008). On the other hand, hypoxic Caco-2 and HT-29 bronchial epithelial cells express the CXCR1 (but not the CXCR2) and undergo chemotactic responses to CXCL8 signaling (Sturm, Baumgart et al. 2005). This could suggest that inflammatory signals upregulate expression of these receptors, but in A549 cells, at least, LPS stimulation does not alter their expression of the CXCR1 or CXCR2 (J. Town, unpublished observation). The blockade of CXCL8 expression in LPS-stimulated epithelial cells by hG31P leads us to speculate that CXCR1 ligands are autocrine inflammatory mediators for these cells. It had been reported previously that CXCL8 is an autocrine growth factor for pancreatic tumour (Takamori, Oades et al. 2000) and melanoma (Schadendorf, Moller et al. 1994; Norgauer, Metzner et al. 1996) cells but, as far as we are aware, this is the first demonstration that ELR-CXC chemokine expression can be a self-perpetuating event for epithelial cells. We also found that simple co-culture of quiescent human neutrophils with A549 cells strongly induced CXCL8 expression in these cultures and that G31P interrupts this response, dramatically reducing chemokine production. We did not determine which population of cells was activated in these co-cultures and therefore responsible for the cytokine expression. A number of mediators released from activated neutrophils (e.g., cathepsin G, elastase, defensins) can induce the release of CXCL8 from respiratory epithelial cells and, reciprocally, a number of epithelial products are neutrophil agonists (Khair, Davies et al. 1996; Skerrett, Liggitt et al. 2004). Although neutrophils can be readily activated by the procedures used in their isolation (Venaille, Misso et al. 1994; Link, Hummel et al. 1997), when cultured alone neither the epithelial cells or neutrophils secreted substantial amounts of CXCL8, indicating that the interactions between these two cells were instrumental in their induction, and that endotoxin contamination of our culture medium by itself would not explain our observations. More importantly, G31P was able to interfere with these intercellular interactions sufficiently to ameliorate their influence on

one another. The remarkable degree to which G31P dampened neutrophilic inflammation in our airway endotoxemic animals suggests that its ability to block the interactions between bronchial epithelial cells and those neutrophils that did find their way into the airways could be another integral component of its therapeutic effects.

Neutrophils are continuously generated in the bone marrow and released into the circulation at exceptionally high rates, which means of course that they need to be equally quickly disposed of if a homeostatic balance is to be maintained. Thus, neutrophils are pre-programmed to undergo apoptotic death quite rapidly, and this is only offset if inflammatory events dictate that there is an extended need for increased numbers of viable cells. A number of inflammatory mediators have anti-apoptotic effects on neutrophils, including the ELR-CXC chemokines (Glynn, Henney et al. 2002; Kobayashi, Voyich et al. 2005), such that cells exposed to these mediators remain viable for extended periods of time and thereby can potentially contribute to the regional anti-microbial activities. This translates also into potential contributions to adventitious pathogenic processes, such that the ability of hG31P to countermand ELR-CXC chemokine-driven anti-apoptotic effects would be of significant benefit to the host by virtue of encouraging functional removal of these cells from the inflammatory environment.

We had previously shown that bovine and a human-bovine chimeric form of G31P are effective in blocking neutrophilic pathology associated with airway endotoxemia (Gordon, Li et al. 2005; Zhao, Li et al. 2007), and we confirmed here for the first time that the human orthologue G31P was similarly effective. Herein we assessed the kinetics of neutrophil infiltration of the airways as well as their activation *in situ* in the lungs. Our data showed that G31P can dramatically reduce neutrophil recruitment and activation, inasmuch as we found high levels of the primary, secondary and tertiary granule markers myeloperoxidase (MPO), lactoferrin (LF), and matrix metalloproteinase-9 (MMP-9), respectively, in lungs of the saline-treated endotoxemic animals, but not in the G31P-treated animals. Previous studies have shown that a number of matrix metalloproteinases are present in the airways during experimental or clinical acute lung injuries or respiratory distress (D'Ortho, Jarreau et al. 1994; Fligiel, Standiford et al. 2006), and we found both MMP-2 and MMP-9 in our BAL samples. While one might argue that the multiple gelatinases detected in our zymograms (i.e., 92 and 68 kD) could have been attributable to

processing of high molecular weight MMP-9 to smaller forms, such processing of MMP-9 reportedly leads to generation of 86 and 82 kD gelatinases (i.e., not a 68 kD activity) (Ramos-DeSimone, Hahn-Dantona et al. 1999). MMP-2 is expressed as a 72 kD zymogen that is processed to a 68 kD gelatinase (Growcott, Spink et al. 2006). MMP-2 is mainly produced by resident cells during acute lung injury, including for example type II epithelial cells or endothelial cells (Fligiel, Standiford et al. 2006), such that the observation that G31P blocked its expression further supports our hypothesis that this antagonist acts not only on the neutrophil, but also on epithelial or other resident cells. Endothelial cells, another source of MMP-2 (Fligiel, Standiford et al. 2006), also express the CXCR2 (Reutershan, Morris et al. 2006), so could potentially also fall under the influence of G31P.

In conclusion, our data indicate that G31P is a potent antagonist of neutrophil activation induced by ELR-CXC chemokines. It targets not only the CXCR1 and CXCR2, but also heterologous GPCR (e.g., CD88/C5aR, BLT1, and FPR) expressed on CXCR1- or CXCR2-positive cells, and it interrupts the autocrine epithelial cell inflammatory cascade triggered by bacterial endotoxins. Thus, the beneficial effects of G31P flow from its ability to simultaneously target multiple inflammatory processes. We speculate that the ability of G31P to antagonize heterologous GPCR may be a very important part of its anti-inflammatory activities, such that ELR-CXC chemokine antagonism in the absence of such activities (e.g., by anti-chemokine antibodies) may provide somewhat less effective protection. An obvious question to ask when one wishes to block neutrophil participation in biological responses is whether there are deleterious effects. For example, in bacterial pneumonia one might expect that these cells may be required for successful elimination of the pathogen(s). However, we have found that G31P very significantly attenuates pathology in aspiration pneumonia but at the same time does not reduce control of the microbial populations that enter the airways (Chapter 5). Blocking neutrophil recruitment in bovine pneumonic pasteurellosis similarly improves outcomes very significantly (Slocombe, Malark et al. 1985). We would predict based on our cumulative results that a broad array of inflammatory diseases would thus benefit from such ELR-CXC chemokine antagonism, as might other processes in which inflammation *per se* is not necessarily prevalent, but in which the ELR-CXC chemokines have nevertheless been implicated.

CHAPTER 5 : BLOCKADE OF NEUTROPHIL RESPONSES IN ASPIRATION PNEUMONIA VIA ELR-CXC CHEMOKINE ANTAGONISM DOES NOT PREDISPOSE TO AIRWAY BACTERIAL OUTGROWTH [3]

Chapter 5 overview

As we showed in the last chapter, hG31P effectively antagonized neutrophilic inflammatory responses to bacterial LPS challenge by virtue of its abilities to block ELR-CXC chemokine signaling through both the CXCR1 and CXCR2 receptors. hG31P also antagonized human airway epithelial cell responses to LPS challenge as well as ligand-induced activation of heterologous GPCRs (e.g., C5aR, BLT1, and FRP), which raise the question of whether hG31P would also inhibit bacterial clearance in the airway. In this chapter we addressed this question. Basically, I set up a guinea pig model of aspiration pneumonia and examined the abilities of hG31P to block neutrophil activation and infiltration into the airway, pulmonary hemorrhagic consolidation, lung tissue chemokine expression, and the airway bacterial burden. Assisted by my co-authors, I designed and finished all the experiments, analyzed the data, and wrote the manuscript.

5.1. INTRODUCTION

Aspiration pneumonia is a condition brought on by aspiration of foreign materials, most often gastric contents. It occurs primarily in unconscious or semiconscious patients, and is relatively common during general surgical anaesthesia (incidence, 1 in 3000) (Olsson, Hallen et al. 1986; Warner, Warner et al. 1993). The local inflammatory sequelae vary from sub-clinical pneumonitis to severe acute lung injury (ALI) and acute respiratory

[3] Zhao Xixing, J. R.Town, F. Li, W. Li, X. Zhang, and J.R. Gordon. Blockade of neutrophil responses in aspiration pneumonia via ELR-CXC chemokine antagonism does not predispose to airway bacterial outgrowth. Submitted to *Pulmonary pharmacology and therapeutics*

distress syndrome (ARDS) (Pepe, Potkin et al. 1982; Fowler, Hamman et al. 1983), depending on the volume and the pH of the gastric contents (Marik 2001). However, ALI/ARDS associated with aspiration pneumonia carries a mortality rate of 10-30% (Olsson, Hallen et al. 1986; Warner, Warner et al. 1993).

Neutrophils are the primary drivers of this inflammatory response in aspiration pneumonia (Folkesson, Matthay et al. 1995; Beck-Schimmer, Rosenberger et al. 2005; Davidson, Knight et al. 2005; Raghavendran, Davidson et al. 2005). When activated, neutrophils release an array of microbicidal factors, including reactive oxygen intermediates (ROI), defensins, and proteolytic enzymes, but they also foster the inflammatory responses through elaboration of proinflammatory cytokines (e.g., TNF, IL-1) and chemokines (e.g., CXCL8/IL-8, CXCL1/GROα). The ELR-CXC motif-containing chemokines (e.g., CXCL8, macrophage inflammatory protein-2 [MIP-2]) are central to the recruitment and activation of neutrophils in such inflammatory settings, including aspiration pneumonia (Folkesson, Matthay et al. 1995; Baggiolini 1998; Rotta, Shiley et al. 2004; Beck-Schimmer, Rosenberger et al. 2005). This suggests that ELR-CXC chemokine antagonism, and thereby amelioration of the neutrophil responses, could potentially offer an adjunct therapeutic approach in aspiration pneumonia. However, an important caveat to such an approach would be that neutrophils are thought to be a critical component of the innate anti-bacterial response. This raises the question of whether blockade of neutrophil recruitment during bacterial infections would be patently detrimental.

The ELR-CXC chemokines chemoattract and activate neutrophils via two closely related G protein-coupled receptors (GPCR), the CXCR1 and CXCR2 (Wolf, Delgado et al. 1998). We have reported that bovine CXCL8$_{(3–73)}$K11R/G31P and human CXCL8$_{(3-72)}$K11R/G31P (hG31P), and human-bovine chimeras thereof, effectively antagonize neutrophilic inflammatory responses to bacterial lipopolysaccharide (LPS) challenge by virtue of their abilities to block ELR-CXC chemokine signaling through both of these receptors (Li and Gordon 2002; Li, Zhang et al. 2002; Gordon, Li et al. 2005; Zhao, Li et al. 2007; Zhao, Town et al. 2009). hG31P also antagonizes human airway epithelial cell responses to LPS challenge as well as ligand-induced activation of heterologous GPCR (i.e., C5a, LTB4, and fMLP) (Zhao, Town et al. 2009) . In the present study, we assessed whether hG31P-dependent blockade of neutrophil participation in the innate immune

response following aspiration of acidified gastric contents would foster bacterial survival/growth in the airway. Our data have shown that the hG31P treatments did not do so and, moreover, they significantly ameliorated pulmonary pathology.

5.2. MATERIALS AND METHODS

5.2.1. Animal and reagents

Most commercial reagents have been described previously (Li and Gordon 2001; Li and Gordon 2002; Zhao, Li et al. 2007) but we also purchased: RNAlater, QIAprep Spin Miniprep, QIAquick Gel Extraction, QIA shredder and RNeasy Mini Kits (QIAGEN Inc, Mississauga, ON); Brilliant qRT-PCR Master Mix Kit (Stratagene Cloning Systems, La Jolla, CA); and blood agar plates (Becton Dickinson). Human G31P was generated and characterized as noted (Zhao, Town et al. 2009). Female Hartley guinea pigs (4-week old, 250-300g) were purchased from Charles River Laboratories (Charles River, MA); all experiments were carried out according to the guidelines established by the Canada Council on Animal Care and were approved by our institutional animal ethics review panel.

5.2.2. Preparation of gastric contents

The protocol used for preparing gastric contents was as described previously (Knight, Davidson et al. 2004; Davidson, Knight et al. 2005) with minor changes. In brief, stomach contents were collected from healthy guinea pig donors, dispersed in normal sterile saline, and filtered through sterile gauze. A stock solution of gastric contents was prepared in normal saline at 100 mg/ml (w/v), aliquoted and stored in -80 ^{o}C. This stock was further diluted to 40 mg/ml and adjusted to pH 2.0 immediately before use.

5.2.3. Guinea pig aspiration pneumonia model

In preliminary experiments we optimized dosing of gastric contents to be used for the challenges, instilling the gastric contents at 0, 5, 10, 20, 35, 50, or 75 mg/kg into the airways of each animal. At 20 h after challenge we harvested the animals and assessed

their pulmonary hemorrhagic consolidation and airway leukocyte responses. The gastric contents dose-dependently induced pulmonary neutrophilic inflammation and hemorrhagic consolidation within the 5-35 mg/kg dose range (data not shown). We similarly assessed the effects of using gastric contents of differing pH and found that a pH of <2.0 was often lethal for the guinea pigs, as were volumes of gastric contents greater than 250 µl. The timing for induction of pulmonary pathology and the G31P treatment doses in this model were also optimized (data not shown).

For the experiments reported herein, we infused 250 µl of acidified gastric contents (35 mg/kg; pH 2.0) into the nares of each experimental animal, and at the same time treated them s.c. with 1 ml of saline alone or containing 250 µg/ml of human G31P. All animals were euthanized at 20 h post-challenge using halothane. The animals were assigned to four treatment groups (n=5/group), as follows: Group 1, pH 7.2 saline i.n. (saline control group); Group 2, pH 2.0 saline i.n. (acidified saline control group); Group 3, acidified gastric contents i.n. and saline treatment s.c.; and Group 4, acidified gastric contents i.n. and G31P treatment s.c.. For the intranasal challenges the animals were anesthetized by i.p. injection of 40 mg/kg ketamine and 5µg/kg xylazine. The experiments were repeated three times, and the depicted data are representative of each experiment.

The respiratory tree of each animal was removed from each animal on euthanasia, gently washed and blotted, and then the dorsal and ventral aspects of each were photographed. The photographic images were scored for the proportion of the surface area that was grossly hemorrhagic using image analysis software. Bronchoalveolar lavage (BAL) fluid collection, BAL WBC (and RBC) enumeration and differentials were then performed as noted (Gordon, Li et al. 2005) and the results expressed as the mean number of cells per ml BAL sample**.** Peripheral blood total WBC counts and differentials were performed as well. BAL fluids and serum were aliquoted and stored at -80˚C, while samples of lung tissue for analysis of chemokine mRNA levels were stored at -20˚C inRNAlater solution until processed for RNA extraction. All samples from each animal were assayed independently.

5.2.4. Myeloperoxidase assay

BAL fluids were diluted 10-fold with 0.5% hexadecyltrimethylammonium bromide (HTAB), and 10 µl of the diluted samples were incubated at room temperature for 10 min with 100 µl tetramethylbenzidine (TMB). The reactions were stopped by addition of 100 µl of 1M phosphoric acid and the MPO levels quantified by spectroscopy (OD, 450 nm). The data expressed as OD_{450} ± SEM.

5.2.5. Lactoferrin ELISA

We employed a standard capture ELISA, with optimized concentrations of capture (2.45 µg/ml) and detection (0.85 µg/ml) antibodies, as noted (Zhao, Li et al. 2007). The standards comprised purified lactoferrin (0 - 400 ng/ml). The BAL samples were diluted 1:10 for the assay, which had a sensitivity of 6.25 ng/ml. The results are expressed as the mean concentration (ng/ml) of each group ± SEM.

5.2.6. Assessment of lung tissue neutrophilia

After bronchoalveolar lavage, the caudal left lung lobe of each animal was fixed on ice for 3 h in acid-alcohol formaldehyde (Gordon, Li et al. 2005), and routinely processed to 7 µm paraffin sections. The tissues were stained with hematoxylin and eosin and examined in a blinded manner at 400x power. The results were expressed as the mean of neutrophils/40× field for each group (n = 5) ± SEM.

5.2.7. Peripheral blood neutrophil chemotaxis

We assessed whether the G31P treatments would block the abilities of the peripheral blood neutrophils to respond to ELR-CXC chemokines. To do so, neutrophils were purified from the peripheral blood of each animal and used for modified Boyden chamber microchemotaxis assays, as reported previously (Li and Gordon 2002; Li, Zhang et al. 2002; Zhao, Li et al. 2007). The purified cells were suspended in phosphate-buffered saline [PBS; pH 7.4] supplemented with 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 5 mM

glucose, and 0.1% bovine serum albumin. Human CXCL8 (10 ng/ml) or PBS were used as chemoattractants for each sample. The assays were quantified by direct counting of at least five 40× objective fields per well. The results are presented as the experimental minus control well values (i.e., CXCL8 minus PBS responses), and expressed as the mean number of cells per 40× field ± SEM.

5.2.8. Isolation of lung tissue total RNA and quantitative real time PCR (qRT-PCR)

RNA from the lung tissue samples was isolated and purified using Qiagen RNeasy Mini Kits, according to the supplier's protocol. The total RNA was quantified spectrophotometrically and stored at -80 °C. The levels of β-actin, CXCL1 and CXCL8 mRNA in each sample were determined by qRT-PCR. The guinea pig gene-specific primers were: β-actin forward: 5'-CGT AAG GAC CTC TAT GCC AAC AC-3' and β-actin reverse: 5'-GAC TCA TCG TAC TCC TGC TTG CT-3'; CXCL1 forward: 5'- CCC AAG AAC ATC CAG AGC GTA G-3' and CXCL1 reverse: 5'- TGG CTT TGC TTC CTT TCA GC-3'; CXCL8 forward: 5'- TGC GAT GCC AGT GTA TTA AGA TT-3' and CXCL8 reverse: 5'- CTC TTC AAG AAC ATG CTC ACC AC-3'. We used a one-step master mix protocol for the qRT-PCR, with a thermal profile of: first segment, 50°C for 30 min and 95°C for 10 min (1cycle); second segment, 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec (40 cycles), followed by a dissociation curve.

5.2.9. BAL fluid bacteriology

BAL fluids were diluted 1:250 after collection and 100µl aliquots were applied to blood agar plates using standard approaches. After overnight incubation at 37°C, the bacterial colonies on each plate were counted, and the colony morphology assessed by microscopic observation. The bacteria from each colony type were also subjected to Gram staining, all by our professional bacteriology service, Prairie Diagnostic Services. The results were expressed as the mean number of Gram-negative or –positive enterobacilli, or Gram-positive corynebacterium-like organisms for the BAL samples from each group (± SEM).

5.2.10. Statistical analyses

The results are expressed as the mean ± SEM. Multiple group comparisons were made using one-way analysis-of-variance (ANOVA), while two-group comparisons were made using student's t-tests (two-tailed).

5.3. RESULTS

5.3.1. Neutrophil blockade in aspiration pneumonia ameliorates pulmonary pathology.

Much of the pathology associated with aspiration pneumonia is attributable to overly aggressive neutrophil responses to the aspirated materials. These can contain acidic particulate matter from the gut in cases of aspirated regurgitate as well as resident upper airway microbes that the aspirate may incidentally wash into the lower airway. While antagonism of the neutrophilic inflammation could potentially be beneficial on one hand, neutrophils are thought to be important for successful clearance of bacteria. Thus, herein we tested the abilities of guinea pigs to successfully handle aspiration of acidified gastric contents when their neutrophil responses to the pulmonary insult were blocked. We used four groups of guinea pigs (n=5/group), including normal saline and acidified saline controls and animals that received the acidified gastric contents with or without treatment with 250 μg/kg recombinant human G31P, as noted in the Materials and Methods. The experiments were repeated three times, with low experiment-to-experiment variance (Table 5.1.); all depicted data are from one experiment that is representative of each experiment.

It is clear that the acidified gastric contents induced strong pulmonary inflammatory responses, including robust airway and tissue neutrophil responses (Fig. 5.1A, B). The challenges also had a significant impact on vascular integrity within the lungs, as there was grossly evident hemorrhagic consolidation across the pleural surfaces (Fig. 5.1C), and we inevitably found substantial numbers of red blood cells (RBC) within the BAL fluids (Fig. 5.1D). The acidified saline control animals displayed pulmonary responses that were indistinguishable from those of pH 7.2 saline-infused controls ($p>0.5$, for each parameter). The airway and tissue neutrophil responses of the aspiration

Table 5.1. Total BAL WBC and PMN data of three independent experiments.

	Total WBC (10^5 cells/ml)			Total PMN (10^5 cells/ml)		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
Saline	5.1	8.7	7.0	0.7	1.9	2.0
GC	19.1	32.9	39.9	11.7	18.7	25.7
GC/G31P	5.2*	17.5	9.5**	1.5*	4.3*	2.4**

* and **, $p \leq 0.05$ and 0.01, respectively, relative to the GC group

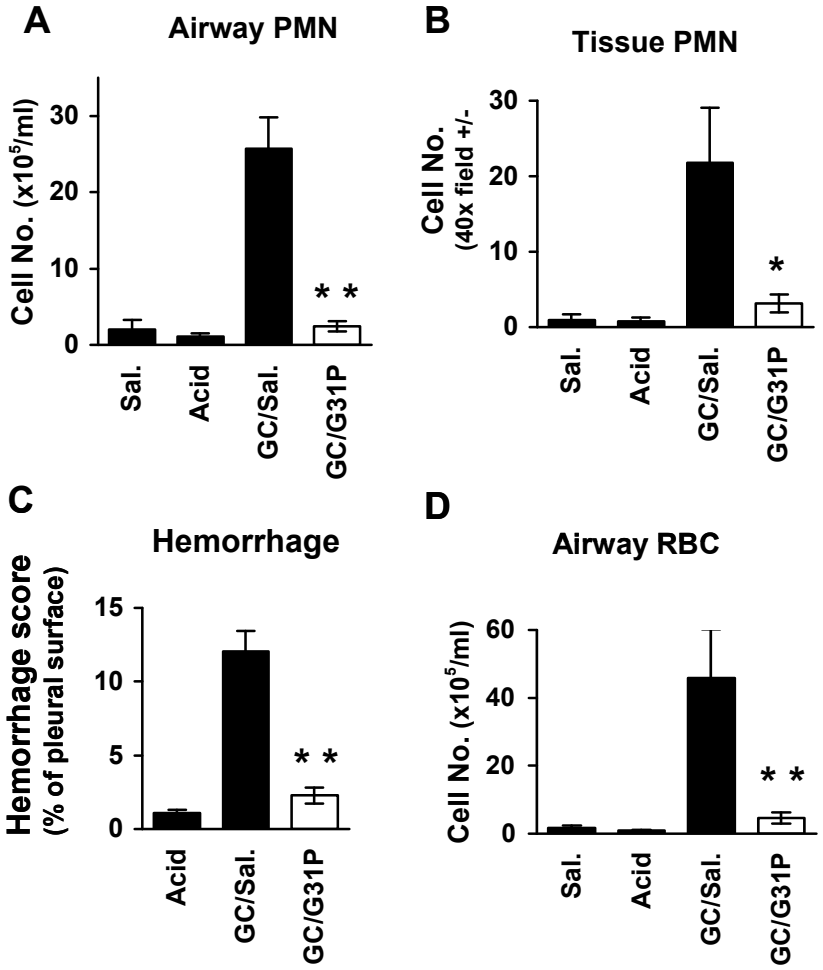


Figure 5.1. Human CXCL8$_{(3-72)}$K11R/G31P can effectively block neutrophil infiltration and loss of vascular integrity in aspiration pneumonia animals.

(**A**) Bronchoalveolar lavage (BAL) white blood cells were enumerated and differential counts were performed for each animal. The neutrophil (PMN) numbers were calculated from this data (Airway PMN), which are expressed as the mean number of cells ($\times 10^5$)/ml (+/- SEM). (**B**) The tissue neutrophil response was assessed by direct counting of haematoxylin & eosin-stained lung tissue sections (Tissue PMN) and the data expressed as the mean number of cells in each 40× object fields (+/- SEM). (**C**) In addition, the BAL red blood cell (RBC) was enumerated as above, and the data were expressed in the same way as airway PMN. The results demonstrated that 250µg/kg G31P significantly reduced the 20 h airway and tissue neutrophil responses ($p<0.01$, and $p<0.05$), and reduced the appearance of red blood cells in the airways ($p<0.01$). Data shown in these figures are from one experiment that is representative of three independent experiments. *, $p<0.05$ or **, $p<0.01$, versus the saline-treated, GC-challenged animal values.

pneumonia animals treated with 250 μg/kg G31P were 90% and 85% reduced, respectively, relative to the saline-treated aspiration pneumonia animals (Fig. 5.1A, B). The G31P treatment also ameliorated that impact of the gastric aspirates on pulmonary vascular integrity, markedly reducing both the pleural hemorrhagic consolidation and appearance of RBC in the airways (Fig. 5.1C, D). Thus, the G31P treatment did effectively block pulmonary neutrophilia and local vascular complications induced by the aspiration of acidic gastric contents.

5.3.2. ELR-CXC chemokine antagonism significantly decreased airway neutrophil degranulation in aspiration pneumonia

While the G31P treatments markedly reduced the pulmonary neutrophil responses, they were not abrogated. Thus we also assessed BAL fluids (BALF) for markers of neutrophil activation within the airways. Degranulation is considered as a hallmark feature of neutrophil activation, such that the levels of neutrophil granule markers in tissue samples are often used as surrogate measures of neutrophilic inflammation. Thus we measured the levels of myeloperoxidase (MPO) and lactoferrin (LF), as indicators of neutrophil primary and secondary granule release, respectively, in the BALF of our experimental animals. G31P treatment of aspiration pneumonia animals reduced the BALF MPO levels to background ($p<0.005$, versus the saline-treated gastic content-challenged group; Fig. 5.2A) and reduced the BALF LF levels by 68% relative to the saline-challenged controls ($p\leq0.05$; Fig. 5.2B). These results demonstrated that the G31P treatments had dramatically reduced neutrophil migration and activation, as determined by direct counting and assessment of activation markers.

5.3.3. Human G31P effectively blocked lung tissue ELR-CXC chemokine mRNA expression

We have shown that G31P treatment inhibited neutrophil activation and migration into lung tissue and airway caused by aspiration of gastric contents. One would predict that while the primary (GC) challenge would upregulate ELR-CXC chemokine expression

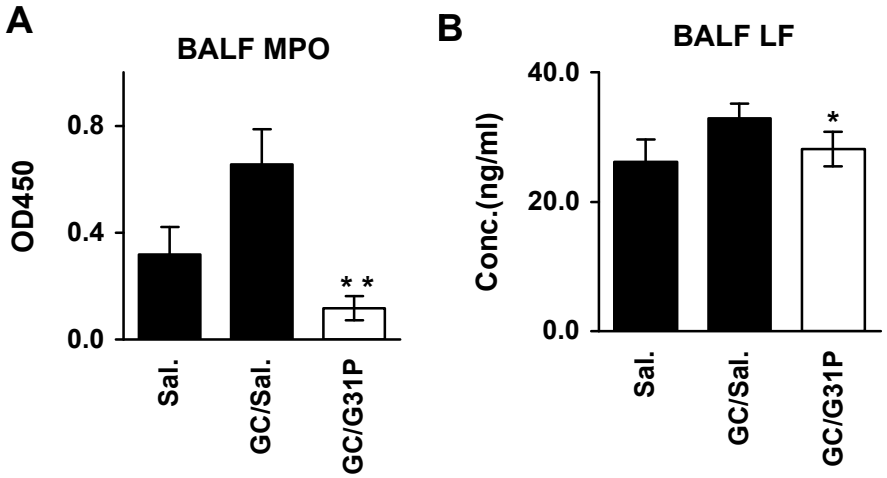


Figure 5.2. CXCL8$_{(3-72)}$K11R/G31P also significantly decreased the appearance of neutrophil primary and secondary granules products in the airway.

Neutrophil primary and secondary granules were assessed by measuring myloperoxidase (MPO) and lactoferrin (LF) using colorimetric assay and ELISA, respectively. (**A**) BAL from each animal was diluted and reacted with the colorimetric substrate TMB, and the developing reaction product was assessed spectrophotometrically, OD_{450}. The data were expressed as the mean OD_{450} (± SEM). (**B**) Diluted BAL was also used to measure the secondary granule, LF using an ELISA assay. The data were expressed as ng/ml (± SEM). The data indicated that G31P significantly decreased MPO production ($p<0.01$) and also LF ($p<0.05$), though to a substantially lesser extent. Data shown in these figures are from one experiment that is representative of four experiments (n=5 animals/group). *, $p<0.05$ or **, $p<0.01$, versus the saline-treated, GC-challenged animal values.

by airway epithelial cells, for example, neutrophil influx into the lungs would both bring additional sources of these chemokines and exacerbate the epithelial response. We have recently found that G31P can partially block ELR-CXC chemokine induction in LPS-challenged human bronchial epithelial cells (footnote 2). We wished therefore to know whether G31P treatments of aspiration pneumonia animals would reduce pulmonary expression of ELR-CXC chemokines and other inflammatory mediators, in addition to blocking neutrophil influx. Thus, we used quantitative real time PCR (qRT-PCR) to probe the lung tissues from our aspiration pneumonia and control animals for expression of CXCL8 and CXCL1 and the cytokines TNFα and IL-1β. We found that even at 20 h after the gastric content challenge, CXCL8 and CXCL1 mRNA levels were still expressed at levels two-fold greater than in saline-challenged (i.e., normal control) animal (Fig. 5.3 A, B). The G31P treatments reduced lung expression of CXCL8 at this time to near background and CXCL1 expression by 50%, relative to the saline control animals (Fig. 5.3A, B). However, at this time TNFα and IL-1β levels were at background in the saline-treated aspiration pneumonia animals, such that the G31P treatment had no discernible effect on their expression (data not shown). These results indicate that G31P treatment did reduce ELR-CXC chemokine expression in the inflamed lung tissues following aspiration of acidified gastric contents.

5.3.4. Neutrophils from G31P-treated animals are hyporesponsive to CXCL8 stimulation *in vitro*

While G31P blocked ELR-CXC chemokine expression in the lungs of our experimental animals, we would predict based on earlier study (Gordon, Li et al. 2005) that, even if exposed to these chemokines *in vivo*, the circulating neutrophils of the G31P-treated animals would be incapable of responding and moving into the tissues. To test this, we purified peripheral blood neutrophils from each animal and assessed their ability to respond in vitro to CXCL8 in chemotaxis assays. As shown in our airway endotoxemia model (Gordon, Li et al. 2005), the cells from the G31P-treated aspiration pneumonia animals were indeed hyporesponsive to CXCL8 (Fig. 5.4A; $p<0.01$, relative to either the normal control or saline-treated aspiration pneumonia animals). This suggested that G31P

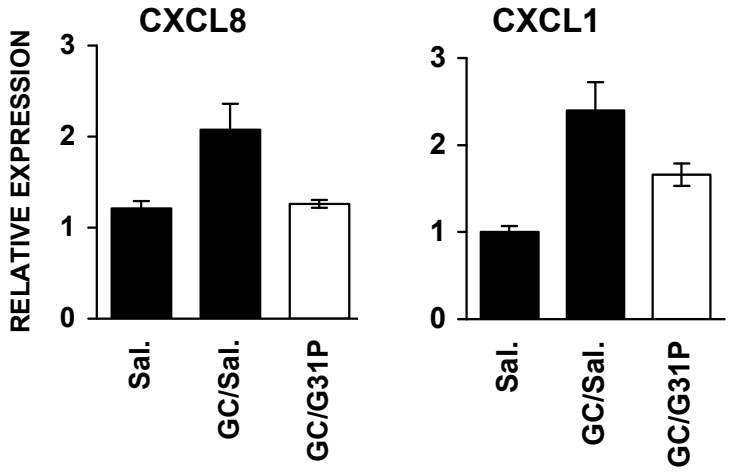


Figure 5.3. CXCL8$_{(3–72)}$K11R/G31P can block ELR-CXC chemokine expression in aspiration pneumonia animals.

Total RNA was purified from lung tissue of each animal and expression of the ELR-CXC chemokines, CXCL8 and CXCL1, was measured using quantitative real-time PCR (qRT-PCR), as described in the *Materials and Methods.* The data were expressed as the relative quantities of mRNA relative to a calibrator sample (a representative saline control group sample). (**A, B**) The result indicate that the aspiration pneumonia animals expressed two-fold more CXCL8 and CXCL1 mRNA relative to saline-challenged aspiration pneumonia animals at 20 after GC challenge, and the G31P can block CXCL8 and CXCL1 mRNA expression by 90% and 50%, respectively.

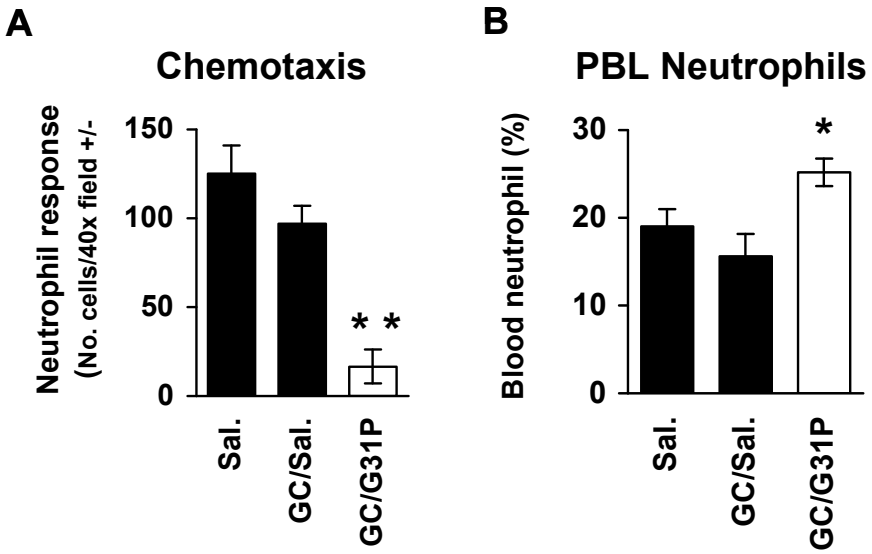


Figure 5.4. CXCL8$_{(3–74)}$K11R/G31P blocks chemotactic responses of guinea pig neutrophils to CXCL8.

As described above, guinea pigs were challenged with saline or GC (+/- G31P) and sacrificed 20 h later, as in Fig5.1. (**A**) Neutrophils were purified from the peripheral blood of each animal and used in modified Boyden chamber microchemotaxis assays in which saline or 10ng/ml human CXCL8 was the chemoattractant. Each sample was assessed in duplicate, and the responses assessed by counting the number of cells within five 40x objective microscope fields of the stained chemotaxis membranes. The data were expressed as the number of cells (experimental minus control) per 40x field (± SEM). The neutrophils from the G31P-treated animals (GC/G31P) were significantly less able to respond to CXCL8 than those from the saline-treated (GC/Sal.) or normal (Sal. $p<0.01$) animals. (**B**) Differential counts were performed on the blood WBC from each group of animals and the proportions of neutrophils enumerated. The data were expressed as the mean percentage of WBC that was neutrophils (± SEM). The data indicate that hG31P treatment modestly increased the proportion of circulating neutrophils in the blood. The data presented are from one experiment that is representative of four experiments (n=5 animals/group). *, $p<0.05$ or **, $p<0.01$, versus the saline-treated, GC-challenged animal values.

had occupied the CXCL8 receptors (i.e., CXCR1 and CXCR2) on the circulating neutrophils in these animals, preventing them from responding to CXCL8 after purification. In addition, we assessed the impact of the various treatments on the circulating neutrophil in our animals, and found that G31P-treatments significantly increased the numbers of neutrophils trafficking in the blood (Fig. 5.4B) relative to the saline-treated aspiration pneumonia animals, which were slightly neutropenic relative to the normal control animals. Others have reported though that surgical anaesthesia can induce a mild neutropenia in healthy control animals (Morisaki, Aoyama et al. 1998).

5.3.5. Neutrophil blockade did not increase bacterial growth in the lungs of animals with aspiration pneumonia

Given the roles of neutrophils in anti-bacterial defenses, a critical element of this investigation was whether neutralization of the pulmonary neutrophil response with G31P would predispose the animals to development of runaway airway bacterial growth. Thus, we assessed the bacterial loads in the BAL fluids of our experimental animals, grossly differentiating the BAL flora as gram-negative or -positive enterobacilli (gr^- or gr^+-entero., respectively), or gram-positive corynebacterium-like (coryne-like) organisms. For each of these bacterial designations, the mean value of bacterial CFU was higher in the saline-treated aspiration pneumonia animals than the G31P-treated animals, although not significantly so (Fig. 5.5.). This data indicates that neutrophil blockade did not negatively affect bacterial clearance in this model.

5.4. DISCUSSION

We have previously generated multiple forms of ELR-CXC chemokine antagonists, including bovine, human, and human-bovine chimeric orthologues (Li and Gordon 2002; Li, Zhang et al. 2002; Gordon, Li et al. 2005; Zhao, Li et al. 2007). Each of these takes on the form of an amino-truncated CXCL8 molecule with arginine and proline substitutions at amino acids K11 and G31, respectively. *In vitro*, each inhibits the responses of normal resting human neutrophils to human CXCR1 and CXCR2 ligands (e.g., CXCL1, CXCL5,

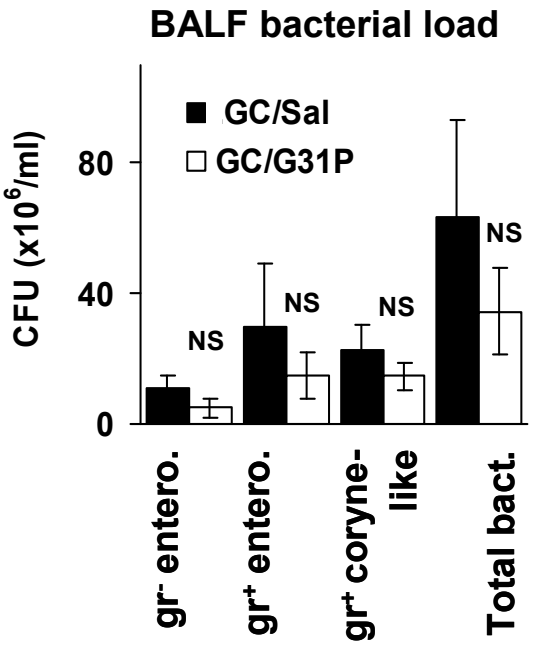


Figure 5.5. Neutrophil blockade did not increase bacterial growth after CXCL8$_{(3–72)}$K11R/G31P treatment.

Aspiration pneumonia was induced in guinea pigs as in Figure 5.1. BAL fluids from each animal were diluted 1:250 and cultured overnight on blood agar plates. The bacteria colonies that developed were differentiated and counted. The data are expressed as the number of colonies ($\times 10^6$) per ml BAL. The result show that three primary groups of organisms, including, gram-negative enterobacilli (gr^- enter rod), gram-positive enterobacilli (gr^+ non-enter rod), and gram-positive corynebacterium-like (gr^+ corynebacterium) were cultured from the BAL. The G31P treatment did not increase the bacterial load in the aspiration pneumonia animal ($p>0.05$). This data is from one experiment that is representative of four experiments (n=5 animals/group) performed.

and CXCL8), including intracellular Ca^{2+} flux, ROI production, and chemotaxis (Li and Gordon 2002; Zhao, Li et al. 2007), as well as their anti-apoptotic activities (Zhao, Town et al. 2009). In addition, we have found that human $CXCL8_{(3-72)}$K11R/G31P (hG31P) antagonizes intracellular Ca^{2+} flux and chemotaxis induced in neutrophils by ligands for heterlogous GPCRs, including LTB4, C5a, and bacterial tripeptide fMLP (Zhao, Town et al. 2009). *In vivo*, each of these antagonists can dramatically reduce pulmonary neutrophilic inflammation, hemorrhagic consolidation and pyrexia in guinea pigs suffering from airway endotoxemia (Gordon, Li et al. 2005; Zhao, Li et al. 2007) including, in the case of the bovine antagonist at least, when administered after the onset of symptoms (Gordon, Li et al. 2005).

In the present study, we examined the ability of human G31P to inhibit pulmonary inflammatory responses when guinea pigs were challenged via the airway with acidified stomach contents, as opposed to purified bacterial endotoxin alone. It is apparent that either the gastric contents themselves contained bacteria or that intranasal infusion of these contents washed upper airway microorganisms into the lower airways. Thus, the insult included bacterial challenge in a highly acidic (pH 2.0) environment. This combination has been used by others as a model of aspiration pneumonia (Marik 2001). We observed inhibition by G31P of pulmonary neutrophil recruitment, hemorrhagic consolidation and ELR-CXC chemokine expression in response to the aspiration of these gastric contents, and found that this G31P treatment did not negatively affect bacterial clearance by the innate immune system. Indeed, if anything, there was a trend towards augmented bacterial clearance in the G31P-treated animals. The residual anti-bacterial defenses that could have provided protection against the gastric content challenges include both physical and adaptive responses (e.g., the mucociliary clearance escalator and epithelial defensin production), although we did not experimentally address these systems in this report.

Neutrophils are major contributors to defensin expression in inflamed airways, wherein they secrete human neutrophil peptides-1, -2, and -3 (Ashitani, Mukae et al. 1998). Indeed, in patients with diffuse panbronchiolitis, BAL neutrophil defensin levels correlate significantly with IL-8 expression and neutrophil numbers (Ashitani, Mukae et al. 1998). Neutrophil serine proteases and defensins also directly affect the airway epithelium, decreasing ciliary beat and increasing mucus production and expression of IL-8 by the

epithelial cells, which would serve to further augment the neutrophil responses (Hiemstra, van Wetering et al. 1998). Thus, G31P would have had protective effects derived directly from blockade of primary neutrophil influx, but also at the level of the epithelial cell by virtue of preventing the neutrophil-dependent activation of these cells.

The accidental aspiration of gastric content is associated clinically with serious illness, including acute lung inflammation (ALI) and/or acute respiratory distress syndrome (ARDS), often with lethal outcomes even for individuals under intensive care (Fowler, Hamman et al. 1983; Olsson, Hallen et al. 1986). Acute lung injury induced by acidic gastric contents is characterized by enhanced chemokine expression and neutrophil sequestration (Raghavendran, Davidson et al. 2005), and others have reported that even sterile acidic solutions can induce lung injury associated with dramatic increases in IL-8 expression and neutrophil activation (Folkesson, Matthay et al. 1995). It was interesting then that, in our model, instillation of 250µl of pH 2.0 saline did not lead to significant neutrophil sequestration or other pathology discernible using our approaches (Folkesson, Matthay et al. 1995; Raghavendran, Davidson et al. 2005). This may have been because our challenge medium was not as acidic as that used by some others or that we used smaller volumes. Others have reported that solutions of pH < 2.5 and gastric content volumes of ≤0.3 ml/kg body weight are required to develop lung injury (Exarhos, Logan et al. 1965; James, Modell et al. 1984). We chose a pH of 2.0 and a gastric content dose of 35 mg/kg because, when combined, these parameters had led to very significant pathology in our preliminary experiments. As our data shows, this included elevated pulmonary neutrophil infiltration and hemorrhagic consolidation, which confirms that these parameters were sufficient to adequately model aspiration pneumonia.

We have assessed the kinetics with which different neutrophil granule markers appear in the airways in endotoxemic animals, and found that the levels of primary (MPO) and secondary (LF) granule markers peaked at different times. Nevertheless, G31P effectively inhibited both in these animals (Zhao, Town et al. 2009). Herein we found that high levels of MPO could still be detected at 20 h after challenge with acidified gastric contents, while LF levels were not markedly different in the pneumonic animals compared with the normal controls. However, both markers were significantly affected by the G31P treatment. It should be noted that myeloperoxidase is also a product of monocytes (Lau

and Baldus 2006). The LF result could reflect the fact that LF is primarily an acute phase reaction product in aspiration pneumonia. In addressing the mechanisms by which G31P inhibited neutrophilic inflammation in this model, we also assessed the pulmonary expression of the ELR-CXC chemokines CXCL1 and CXCL8. It has been reported that in a mouse model of aspiration pneumonia expression of macrophage inflammatory protein (MIP)-2 and keratinocyte-derived cytokine (KC) peaked at 6 h, though both were still elevated relative to normal controls at 24 h after challenge (Raghavendran, Davidson et al. 2005). In our study, we detected elevated levels of CXCL1 and CXCL8 expression in our animals at 20 h, and G31P inhibited expression of both chemokines. This confirms that G31P had local effects on chemokine expression in the lungs, perhaps as noted above by simply reducing neutrophil-dependent exacerbation of the epithelial cell responses. When we addressed the more systemic outcomes of the G31P treatments, we found apparently increased numbers of circulating neutrophils in the G31P-treated aspiration pneumonia animals relative to the saline-treated ones. This is likely attributable to G31P's blockade of the ELR-CXC chemokine receptors on these cells, prevent them from marginating in the pulmonary capillary bed under the influence of the locally expressed chemokines (e.g., CXCL8). The circulating neutrophils of our G31P-treated animals were hyporesponsive to CXCL8, as noted previously in airway endotoxemia (Gordon, Li et al. 2005).

In clinical settings, it is common to find gram-positive cocci and gram-negative bacilli in transtracheal samplings from aspiration pneumonia patients (Marik 2001). These bacteria arise mainly from the nasopharynx or oropharynx, and enter the lung with the aspirated regurgitate (Marik 2001). The aspirate volume and bacterial burden, and the general condition of the individual's innate immune system correlate strongly with aspiration pneumonia pathology (Marik 2001). In our model we instilled gastric contents intranasally, which would have effectively flushed the nasopharyngeal and tracheal flora directly into the lungs. We found that the G31P treatments did not increase the bacterial loads in the lungs of our animal. It had been reported that the inhibition of MIP-2 bioactivity *in vivo* impairs *Klebsiella pneumoniae* clearance in a mouse model, although the kinetics of the inflammatory response in that model and ours were quite different (e.g., they observed maximal MIP-2 expression at 48 h) (Standiford, Kunkel et al. 1996). In our system, bacterial clearance was not affected by the inhibition of neutrophil function, which

may be because the bacteria in the airways of our animals were mostly likely normal commensals from the nasopharynx or oropharynx, as opposed to pathogenic organisms (e.g., *K. pneumoniae*). Respiratory commensals may normally become pathogenic only adventitiously (e.g., when confronting a compromised immunoinflammatory system).

It has been reported that aspiration of acidic regurgitates can damage both ciliated and non-ciliated tracheal cells, which would be anticipated to affect mucociliary clearance (Gaynor 1988). It may also decrease airway epithelial expression of β-defensin-1, but not β-defensin-2 (Nakayama, Jia et al. 2002). On the other hand, the abilities of alveolar macrophage to phagocytose tracer particles is significantly enhanced when they were exposed to acid environments *in vitro* or *in vivo* (Naumann and Schlesinger 1986). In our system, bacterial clearance was not affected by antagonism of neutrophil function, suggesting that alternate pulmonary microbicidal functions (e.g., macrophage phagocytosis, β-defensin expression) were sufficient to deal with the insult.

In summary, our data indicate that our ELR-CXC antagonist, G31P, can effectively reduce aspiration pneumonia pathology in a guinea pig model of disease, and thereby suggest that broad spectrum ELR-CXC chemokine antagonism may be useful approach in treating patients who have aspired regurgitates.

5.5. ACKNOWLEDGEMENTS

We would like to acknowledge the kind assistance with bacteriology provided by Ms. Fei Huang and Dr. Manuel Chirino-Trejo (Department of Veterinary Microbiology, University of Saskatchewan).

CHAPTER 6 : REDUCTION IN LOCAL AND REMOTE ORGAN INJURY BY ELR-CXC CHEMOKINE BLOCKADE IN SUPERIOR MESENTERIC ARTERY ISCHEMIA/REPERFUSION INJURY [4]

Chapter 6 overview

To extend our knowledge of the effects of hG31P on other neutrophil-mediated inflammatory diseases, in this chapter I set up a rat superior mesenteric artery (SMA) ischemia and reperfusion (I/R) injury model and assessed the roles of neutrophil and ELR-CXC chemokines in local (i.e., gut) and remote (e.g., lung) organ pathology. I also examined the effects of G31P on pulmonary neutrophil sequestration, pulmonary vascular permeability, and gut pathology. This manuscript mainly describes the process of my setting up the intestinal I/R injury model and the characterization of the antagonist activities of G31P in reducing local and remote organ injury. I set up this model with assistance of Ms Jennifer Town, Xiaobei Zhang, and Aimei Yang, designed and finished all the experiments. Finally, I analyzed the results and wrote the manuscripts with the assistance from my co-authors.

6.1. INTRODUCTION

It has been widely accepted that intestinal ischemia/reperfusion (I/R) injury is associated with severe multiple organ failure (MOF), including the lung (Schmeling, Caty et al. 1989; Caty, Guice et al. 1990; He, Han et al. 2008). A number of proinflammatory cytokines and chemokines are critically involved in this pathology (Caty, Guice et al.

[4] Zhao Xixing, J. R. Town, A. Yang, X. Zhang, G. Sawicki, and J.R. Gordon. Reduction in local and remote organ injury by ELR-CXC chemokine blockade in superior mesenteric artery ischemia/reperfusion injury. Submitted to *Journal of Surgical Research*

1990), although the pathogenesis of intestinal I/R-induced gut pathology and MOF is complex and not completely understood. Nonetheless, reactive oxygen intermediate (ROI) generation and neutrophil sequestration are believed to be two fundamental causative factors (Schmeling, Caty et al. 1989; Souza, Bertini et al. 2004; Cerqueira, Hussni et al. 2005). The pivotal role of neutrophils in these processes (Schmeling, Caty et al. 1989; Bless, Warner et al. 1999; Cerqueira, Hussni et al. 2005) suggests that blockade of their recruitment could be an important therapeutic approach to intestinal I/R injury. The list of neutrophil agonists potentially involved in I/R injury includes TNFα and IL-1β (Caty, Guice et al. 1990) and the ELR-CXC chemokines (e.g., CXCL1, CXCL8, MIP-2) (Schmeling, Caty et al. 1989; Sekido, Mukaida et al. 1993; Bless, Warner et al. 1999), but also C5a (Bless, Warner et al. 1999; Wada, Montalto et al. 2001), LTB4 (Souza, Coutinho et al. 2000), ROI (Koike, Moore et al. 1993), and adhesion molecules (Bless, Warner et al. 1999). Multiple reports have implicated individual ELR-CXC chemokines as primary effectors and shown that their neutralization ameliorates I/R-induced local and remote organ pathology (Bless, Warner et al. 1999; Miura, Fu et al. 2001; Souza, Bertini et al. 2004; Kaneko, Tamura et al. 2007), making these mediators attractive therapeutic targets.

In humans, the ELR-CXC chemokines include growth-related oncogene-α, -β, and -γ (GRO-α, -β, -γ; CXCL-1, -2, and -3, respectively), epithelial neutrophil-activating peptide-78 (ENA-78; CXCL5), granulocyte chemotactic protein-2 (GCP-2; CXCL6), neutrophil-activating peptide 2 (NAP-2; CXCL7), and IL-8 (CXCL8) (Baggiolini 1998). Each of these chemokines binds to either the CXCR1 and/or CXCR2; CXCL6, CXCL7, and CXCL8 bind both receptors, while the others bind only the CXCR2 (Ludwig, Petersen et al. 1997; Wolf, Delgado et al. 1998). Rat ELR-CXC chemokines aren't strictly comparable to the human chemokines, but include CINC-1 (GRO), -2α, -2β, and -3 (MIP-2). Each binds to target cells only via CXCR2, even though homologues of both the CXCR1 and CXCR2 have been discovered in rats (Dunstan, Salafranca et al. 1996; Shibata, Konishi et al. 2000). CXCR1 and CXCR2 belong to the class of receptors called the G protein-coupled receptors (GPCR), as do the receptors for multiple other important inflammatory neutrophil agonists (e.g., C5a, LTB4, platelet activating factor [PAF], and the bacterial tripeptide formyl-Met-Leu-Phe [fMLP]) (Zhelev, Alteraifi et al. 2004).

We recently developed an ELR-CXC chemokine antagonist, human $CXCL8_{(3-72)}$K11R/G31P (hG31P). This CXCL8 analogue can dramatically reduce pulmonary pathology related to airway challenge with bacterial endotoxin (Zhao, Town et al. 2009), but also pathology subsequent to aspiration of bacterial-laden gastric contents (i.e., in aspiration pneumonia) (Chapter 5). We have shown that hG31P antagonizes CXCR1-dependent responses as well as those related to CXCR2-exclusive ligands but, importantly, it also antagonizes heterologous GPCRs (i.e., C5a, LTB4, and fMLP) that are involved in neutrophilic inflammation (Zhao, Town et al. 2009). We hypothesize that hG31P's ability to antagonize both ELR-CXC chemokine receptors as well as these alternate GPCR could potentially provide it with a strong therapeutic advantage during neutrophilic inflammatory events. In this study, we employed a rat model of superior mesenteric artery (SMA) ischemia and reperfusion (I/R) injury and characterized the effects of hG31P on local and remote organ pathology in this model system.

6.2. MATERIALS AND METHODS:

6.2.1. Reagents and Animals

$CXCL8_{(3-72)}$K11R/G31P (G31P) was prepared as described (Zhao, Town et al. 2009). Reagents included buprenorphine analgesic (Reckitt Benckiser Pharmaceuticals Inc, Richmond, VA), ketamine (Bioniche, Bellville, ON), xylazine (Bayer Healthcare, Toronto, ON), isofluorane (Baxter, Mississauga, ON), iodine surgical detergent (0.75% iodine), ocular lubricant (Allergan Inc, Markham, ON), RNAlater, QIA shredder and RNeasy Mini Kits (QIAGEN Inc, Mississauga, ON), and a qRT-PCR one-step master mix kit (Brilliant SYBR® Green qRT-PCR Master Mix Kit; Stratagene, LaJolla, CA). Other reagents that were purchased commercially have been described previously (Li and Gordon 2001; Li, Zhang et al. 2002; Zhao, Li et al. 2007). Male Sprague-Dawley rats (250-300g, 5-week old) were purchased from Charles River Laboratories (Charles River, MA). All the experiments were carried out according to the guidelines established by the Canada Council on Animal Care and were approved by our institutional animal ethics review panel.

6.2.2. Animal model of intestinal I/R-induced injury

Rats were fasted 20 hours before the experiments but allowed free access to water. On the day of the experiment, the animals were anaesthetized using ketamine/xylazine (40 μg/kg/ 5 μg/kg, i.p.) and maintained under anaesthesia with 1% isoflurane and 1% oxygen. The animals were given 0.05 mg/kg buprenorphine i.m. for pain management. A laparotomy was performed and the superior mesenteric artery (SMA) was isolated and totally occlusion for 1 hour using a plastic loop and tubing. After removal of the ligature, the abdominal incision was closed using 4-0 monofilament suture thread. The animals were allowed to recover for 2 hours as the reperfusion injury developed, and then they were euthanized with halothane. Sham-operated animals underwent identical surgical procedures with the exception that the plastic loop and tubing were not closed to occlude the SMA. We ran multiple preliminary experiments in order to optimize ischemia and reperfusion times, including 0.5, 1, 1.5, and 2 h ischemia and 0.5, 1, 2, 4, 8, 16 h reperfusion time. Based on a minimization of mortality with maintenance of discernible pathology, 1 and 2 h were chosen as our ischemia and reperfusion times, respectively, for all subsequent experiments. For these the animals were assigned to three groups: sham, I/R with saline, and I/R with 500 μg/kg G31P s.c. (n=5). The dose of G31P was chosen based on preliminary experiments. All experiments were repeated three times.

6.2.3. Determination of circulating leukocytes and neutrophils

Blood smears were made and stained with Wright's solution and differential counts performed on 200 cells per sample. Total white blood cells (WBC) were enumerated by hemocytometer counting after lysis of the red blood cell (RBC) with 2% acetic acid in water. The total neutrophil numbers were calculated from these values.

6.2.4. Examination of bronchoalveolar lavage (BAL) fluid protein levels, neutrophil and RBC numbers.

Bronchoalveolar lavage (BAL) fluid was collected as noted previously using 2 ml volumes of sterile saline (10). BAL total WBC and RBC were enumerated by direct

counting, and the results expressed as the mean number of cells per BAL sample (± SEM). BAL WBC cell differentials were determined using Wright's solution-stained BAL cell cytospin preparations and the total numbers of neutrophils were calculated using these data. All the data are expressed as means ± SEM. The total protein level was determined using duplicate replicates in a Bradford microassay according to the suppliers protocols (Bio-Rad Laboratories), with bovine serum albumin protein standards. The data was expressed as μg protein/ml BAL fluid (± SEM).

6.2.5. Measurement of MMP-2 and MMP-9 by zymography

Lung and gut tissue homogenates were prepared and MMP-2 and -9 levels were assessed as described previously (Sawicki, Leon et al. 2005; Yu, Yang et al. 2006). Briefly, equal weights (100 mg wet wt) of right lung and jejunum from each animal were suspended in 1 ml of 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM phosphate buffer (pH 6.0) and homogenized for 20 seconds, then sonicated three times for 30 seconds each, all on ice. The homogenates were centrifuged at 12,000 rpm at 4°C and the supernatants harvested and stored at –80°C. Next, 40 μg equivalents of homogenate were applied to 8% polyacrylamide gels containing 2 mg/mL gelatin. After electrophoresis, the gels were first rinsed 3 times for 20 minutes each in 2.5% Triton X-100, then twice for 20 min each in incubation buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 150 mmol/L NaCl, and 0.05% NaN_3), and finally soaked overnight at 37°C in incubation buffer. The soaked gels were stained for 2 h (2% Coomassie Brilliant blue G in 25% methanol/10% acetic acid [w/v]) before destaining with 2% methanol/4% acetic acid. The gelatinase activities of the samples were discerned as transparent bands against the Coomassie blue-stained gelatin background. To quantify the activities of enzymes, these zymograms were imaged using a GS-800 Calibrated Densitometer (Bio-Rad). The intensities of the individual bands were analyzed using Quantity1 software (Bio-Rad) and reported as such. Conditioned medium from untreated HT1080 cells was used as MMP-2 and MMP-9 reference standard (Tanaka, Iwamoto et al. 1995).

6.2.6. Measurements of tissue cytokine and chemokine mRNA levels using quantitative real-time PCR (qRT-PCR).

Lung and jejunum tissues were harvested and stored in 600 µl RNAlater at -20°C. The sample RNA was later purified using commercial RNA preparation kits, according to the supplier's protocol. The total RNA was quantified spectrophotometrically and stored at -80 °C. The levels of β-actin, TNFα, IL-1β, IL-6, IL-10, GRO, CINC-2a, and MIP-2 mRNA in each group were determined by qRT-PCR, using the rat gene-specific primers (Table 6.1.). We used a one-step qRT-PCR master mix kit with a thermal profile of: first segment, 50°C for 30 min and 95°C for 10 min (1 cycle); and second segment, 95°C for 30 sec, 62°C for 30sec, and 72°C for 30 sec (40 cycles), followed by a dissociation curve.

6.2.7. Evaluation of lung tissue neutrophil sequestration

After bronchoalveolar lavage, the caudal left lung lobe of each animal was fixed for 3 h in acid-alcohol formaldehyde (Gordon, Li et al. 2005), and routinely processed to 6 µm paraffin sections, which were stained with hematoxylin and eosin. Neutrophils were enumerated by microscopy in a blinded manner under a 40× objective. The results were expressed as the mean of neutrophils/40× field for each group (n = 5) ± SEM.

6.2.8. Assessment of jejunal and pulmonary pathology.

After euthanasia, the intestinal track of each animal was examined and grossly graded for signs of edema and discolouration. In addition, the contents of the gut were gently extruded and examined. We established a five-point scoring system to assess gross lung pathology, as follows: 0, no discernible edema, discolouration of gut wall or jejunal contents; 1, no discernible edema or discolouration, but jejunum contains yellow fluid; 2, 10% of jejunum edematous, discoloured, and contained bloody-looking fluid; 3, 25% of jejunum edematous, discoloured, and contained bloody-looking fluid; 4, 50% of jejunum edematous, discoloured, and contained bloody-looking fluid; 5, 100% of jejunum edematous, discoloured, and contained bloody-looking fluid. To assess histopathology, the caudal left lung lobe of each animal and jejunum wall were fixed and processed as

Table 6.1. Rat qRT-PCR primers employed for assessing tissue cytokine and chemokine expression.

Target genes	Upstream primers (5'-3' orientation)	Downstream primers (5'-3' orientation)
β-actin	AGAGGGAAATCGTGCGTGAC	CGATAGTGATGACCTGACCGT
TNFα	TCGAGTGACAAGCCCGTAGC	GAAGAGAACCTGGGAGTAGATAA
IL-1β	GATGATGACGACCTGCTAGTGTG	TGGCTTATGTTCTGTCCATTGAG
IL-6	ACAGAGGATACCACCCACAACAG	AACTCCAGAAGACCAGAGCAGATT
IL-10	TTTCTGGGCCATGGTTCTCT	CCTTACTGCAGGACTTTAAGGGTTA
GRO	ATCCAGAGTTTGAAGGTGATGC	CGACCATTCTTGAGTGTGGCTAT
CINC-2α	GCTTCTGCTGCTTCTGCTGATG	TGGCTATGACTTCTGTCTGGGTG
MIP-2	AACATCCAGAGCTTGACGGTGAC	GCCTTGCCTTTGTTCAGTATCTT

described above, stained with hemotoxylin and eosin (H&E) and examined in a blinded manner at 400× magnification.

6.2.9. Statistical analyses

The results are expressed as the mean ± SEM. Multiple groups' comparisons were made using one-way analysis-of-variance (ANOVA) and two-group comparisons were made using student's t-tests (two-tailed).

6.3. RESULTS

6.3.1. The superior mesenteric artery ischemia/reperfusion injury model in rats

We surgically induced intestinal ischemia for one hour by ligature of the superior mesenteric artery (SMA), and then removed the clamp to allow reperfusion of the affected tissues. A vascular clamp was set in place around the artery also for one hour in the sham I/R injury animals, but not tightened to occlude blood flow. Two hours after surgical closing of the abdominal cavity we assessed the impact of these challenges on the animals (Fig. 6.1.). On gross examination, the jejunum of the sham I/R injury animals was a healthy pink colour, firm and largely empty of contents (pathology score, 0.3). On the other hand, the jejunum of the I/R injury animals was dark red-purple in colour and edematous, fragile to the touch, and contained a substantial volume of bloody fluid (pathology score, 3.5). Histologically, the mucosal epithelium within the jejunum of the sham I/R injury animals was intact, as were the villi, while the jejunal epithelium of the saline-treated I/R injury rats were entirely destroyed (Fig. 6.2.). The damage to the villous processes of the I/R injury animals extended deep into the crypts such that very little villous architecture remained. There were no inflammatory cells within the lamina propria of the sham-surgical animals. The residual lamina propria of the I/R injury animals contained modest numbers of necrotic inflammatory cells and these appeared also within the amorphous contents of the gut lumen.

On gross examination of the remaining organ systems of our animals, we found that in the I/R- injury animals the pleural surface of the lungs appeared slightly hyperemic,

but otherwise healthy, while all other organ systems appeared healthy. We did bronchoalveolar lavages (BAL) on each animal and found few neutrophils in the BAL fluids of the SMA I/R-injury animals (~1% of WBC, data not shown). However, significant numbers of neutrophils were found in lung tissues of the I/R-injury, but not sham surgery animals ($p \leq 0.01$; Fig. 6.1B). As a further assessment of the extent of pulmonary involvement in SMA I/R injury pathology, we also assessed transcapillary vascular leakage in the lungs, using BAL fluid protein levels and red blood cell (RBC) numbers as surrogate markers (Middelveld and Alving 2001). We found significantly more protein in the BAL of the experimental animals than the sham surgical controls ($p \leq 0.01$; Fig. 6.1C) and also increased numbers of RBC (Fig. 1D).

6.3.2. ELR-CXC chemokine antagonism ameliorates local and distant organ pathology in SMA I/R injury

A large number of inflammatory mediators have been implicated in I/R injury pathology, including the ELR-CXC chemokines (Caty, Guice et al. 1990; Sekido, Mukaida et al. 1993; Miura, Fu et al. 2001). For that reason we tested the impact of broadly antagonizing the ELR-CXC chemokines expressed in our I/R injury animals by systemic delivery of $CXCL8_{(3\text{-}72)}$K11R/G31P (G31P). G31P treatment at the time of ischemia induction had a sparing effect on loss of jejunal integrity induced by I/R injury. The extent of the gross jejunal pathology, as determined by the tissue edema, discolouration, and the lumen contents, was modestly lower in the G31P-treated animals (pathology score, 2.6) compared to the saline-treated I/R injury cohort (Fig. 6.1A). Histologically, in contrast to the saline-treated I/R injury animals, much of the mucosal epithelium and villous architecture in the G31P-treated animals was intact, although the tips of some villi were damaged and the lumen contained modest amounts of amorphous material (Fig. 6.2).

As the jejunal mucosa and crypts were largely destroyed by the I/R injury in the saline-treated group, we were unable to directly assess neutrophil involvement in this pathology. We therefore assessed the levels of myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9), markers of neutrophil primary and tertiary granule release, respectively, within the gut tissue. The results show that the levels of these two markers

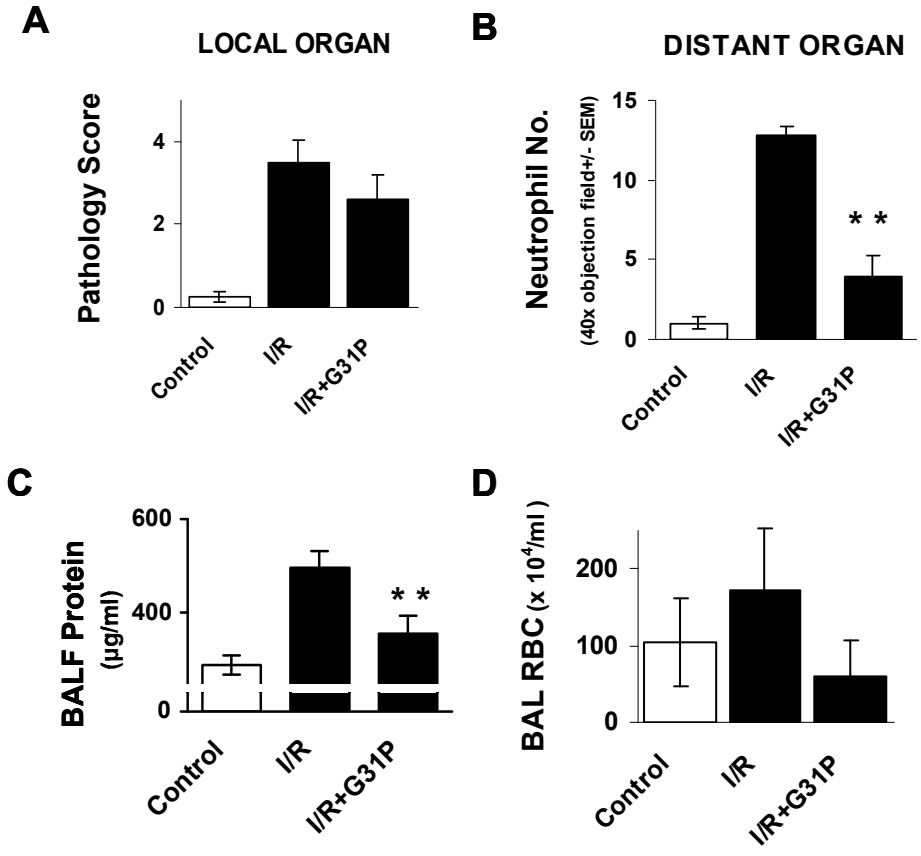


Figure 6.1. G31P can prevent local (gut) and remote (lung) tissue injury after superior mesenteric artery ischemia and reperfusion.

Ischemia-reperfusion injury was induced in male Sprague-Dawley rats (n=5/group) by total occlusion of the superior mesenteric artery (SMA) for 1hour followed by reperfusion for 2 hour. At this time sham-surgical rats (control), saline-treated ischemia-reperfusion animals (I/R), and G31P-treated I/R animals were sacrificed. **(A)** The gross levels of jejunum (local organ) pathology was determined by direct examination of the gut tissues (hemorrhage, edema), as noted in the *Material and Methods*. **(B)** The numbers of neutrophils present in lung tissue sections were enumerated by direct counting at 400x power, while **(C)(D)** the bronchoalveolar lavage fluid (BALF) levels of protein (µg/ml) and red blood cell (RBC) numbers were assessed by protein microassay and direct counting as surrogate measures of pulmonary vascular leakage. The mesenteric I/R dramatically augmented gut pathology, neutrophil infiltration of the lung tissues, and loss of vascular integrity in the lungs, and the G31P treatments significantly reduced these effects. The result is representative one of two independent experiments. **, $p \leq 0.01$, relative to the saline-treated I/R-operated rats.

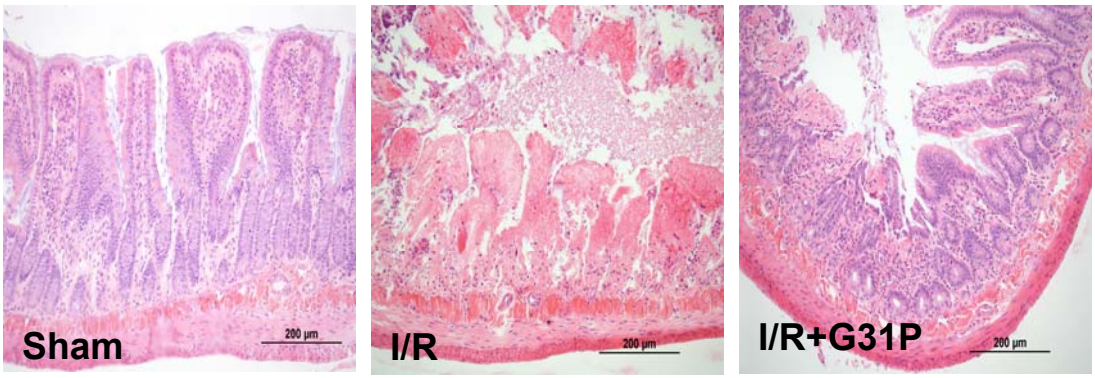


Figure 6.2. Photomicrographs of the jejunal injury in sham-surgical and saline- or G31P-treated I/R injury animals.

The jejunum from all the animals in Fig. 6.1 were processed to paraffin sections, stained with standard H& E stain, and representative sections photographed. The sham surgery animals did not suffer any appreciable jejunal pathology (left panel). On the other hand, the villie and crypts of the I/R animals suffered massive loss of integrity (middle panel), and G31P treatment had very significant sparing effects on this I/R-I-induced mucosal and crypt erosion (right panel). Original magnification × 200.

increased in the jejunal tissues of the I/R injury animals (Fig. 6.3A, B) and that the G31P treatment reduced the levels of both by 70-80%. We also assessed the levels of MMP-2 (a product of activated epithelial cells(Fligiel, Standiford et al. 2006)), and found that I/R injury induced a three-fold increase of the levels of MMP-2 in the gut, while the G31P treatment reduced its expression by ≈50% (Fig. 6.3B).

Antagonizing the ELR-CXC chemokines also had protective effects on the distant organ pathology in our animals. The numbers of lung parenchymal neutrophils was ≈70% lower in the G31P-treated animals than in the saline-treated I/R injury animals ($p \leq 0.01$; Fig. 6.1B). And this reduction in inflammatory cell involvement in the G31P-treated animals was reflected by a similar reduction in transcapillary vascular leakage (BAL fluid protein levels and RBC numbers; Fig. 6.1C, 1D). Compared with the levels of MMP-9 and -2 in the jejunum, there were very low levels of these two gelatinases in the lung tissues, such that the zymogram signals were not easily discernible (data not shown). We also assessed whether I/R injury led to a peripheral neutrophilia in our animals, but found that the total WBC and neutrophil counts were not significantly elevated in the I/R injury animals (Fig. 6.4A, 4B, 4C). We did however observe a significant increase in the percentage of circulating neutrophils in the G31P-treated I/R injury animals, relative to the saline-treated I/R injury animals (Fig. 6.4B). Taken together, our data confirm that distant organ involvement was a significant event in our model of SMA I/R injury and that ELR-CXC chemokine antagonism significantly reduced this pathology.

6.3.3. ELR-CXC chemokine blockade reduces local and remote organ inflammatory mediator expression

Our G31P treatments reduced neutrophil influx into the affected tissues and also reduced the gut pathology scores, though somewhat less dramatically so. We wished to determine whether these effects were correlated with local expression of inflammatory cytokines and chemokines. Thus we measured the tissue levels of TNFα, IL-1β, IL-6, IL-10, GRO, CINC-2α, and MIP-2 expression in both the gut and lungs of our animals using qRT-PCR. In the jejunum, I/R injury markedly induced expression of IL-6, GRO, CINC-2α and MIP-2, but only modestly upregulated expression of TNFα (Fig. 6.5.). IL-1β was

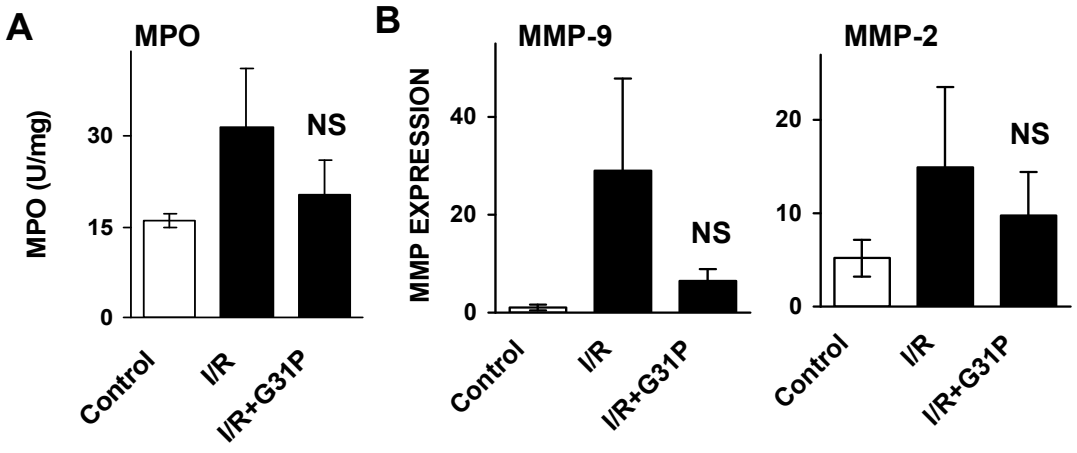


Figure 6.3. G31P treatments modestly ameliorate local neutrophilic inflammation in I/R injury animals.

The jejunal tissues of the animals in Fig. 6.2 were homogenized and the levels of neutrophil myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9), as well as MMP-2 were assessed by colorimetric assay (MPO) and gel zymography (MMP-9 and MMP-2). MPO and MMP-9 were employed as surrogate measures of neutrophil influx into these tissues, the integrity of which was almost completely compromised. All three markers were increased in the I/R injury animals and G31P treatment reduced each of them. The MMP-9 and -2 levels are presented as relative units. The results depicted are from one experiment that is representative of two independent experiments.

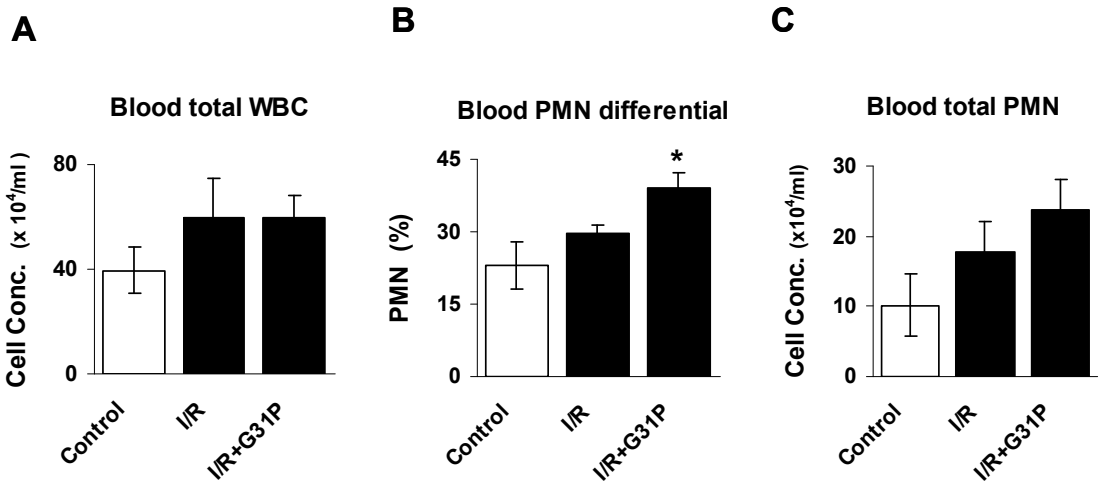


Figure 6.4. Impact of G31P treatments on circulating neutrophils in I/R injury animals.

Peripheral blood from the animals in Fig. 1 were obtained and **(A)** the numbers of white blood cells (WBC)/ml blood were determined by direct counting using a hemocytometer. Blood smears were generated from this blood and stained with Wright's solution, then **(B)** the proportion of neutrophils in the WBC were determined by direct counting, then **(C)** the total blood neutrophils calculated using this data. The data are expressed as the mean number of WBC, percent neutrophils, and numbers of neutrophils (± SEM), respectively. The data indicate that G31P treatment did not change total WBC, but slightly increased total neutrophils in the blood versus saline treatment led to a significant increase in the proportion of circulating neutrophils. These results are from one experiment that is representative of two independent experiments. *, $p<0.05$, versus the saline-treated, I/R-injury animals.

not significantly upregulated as a consequence of the I/R injury. ELR-CXC chemokine blockade decreased IL-6 and IL-10 expression by 20% and 44% respectively, but had no discernible effect on TNFα expression. Interestingly, the G31P treatment also reduced MIP-2 and GRO, but not CINC-2α, expression.

In the lungs, we observed markedly reduced inflammatory mediator expression relative to the gut, with some of these not achieving expression levels two-fold greater than the sham surgical controls (our internal cut-off for significantly increases in qRT-PCR signals). Thus, we consider that there were no significant increases in TNF, IL-6, GRO, or CINC-2α expression in the I/R injury animals relative to the sham surgical controls (data not shown). On the other hand, the levels of IL-1β, MIP-2, and IL-10 were increased approximately three-fold with the tissue injury. G31P treatments reduced the expression of MIP-2 to near background and reduced IL-1β and IL-10 expression by 50 and 30%, respectively, relative to the saline control animals (Fig. 6.6.). These results indicate that G31P treatment did reduce ELR-CXC chemokine expression in the inflamed lung tissues following intestinal ischemia and reperfusion.

6.4. DISCUSSION

Trauma-associated multiple organ failure (MOF) is a recognized sequel to intestinal ischemia-reperfusion (I/R) injury, and neutrophil sequestration is known to play a critical role in mediating both local and remote organ dysfunction (Schmeling, Caty et al. 1989; Koike, Moore et al. 1993; Cerqueira, Hussni et al. 2005). Therapies for such MOF have focused on blocking neutrophil recruitment, with positive experimental outcomes being realized through antagonism of individual ELR-CXC chemokines. Simultaneous targeting of two of these chemokines (e.g., GRO and MIP-2) provides superior protection over targeting only one (Miura, Fu et al. 2001). On the other hand, antagonizing alternate neutrophil ligands (e.g., C5a, LTB4, and PAF) reportedly only partially inhibits neutrophil-mediated I/R injury (Souza, Cara et al. 2000; Souza, Coutinho et al. 2000; Arumugam, Shiels et al. 2002; Kohtani, Abe et al. 2002). Despite the reports of a critical role for neutrophils in intestinal I/R injury, there are reports that depleting circulating neutrophils before experimental induction of I/R injury does not reduce either local or remote organ

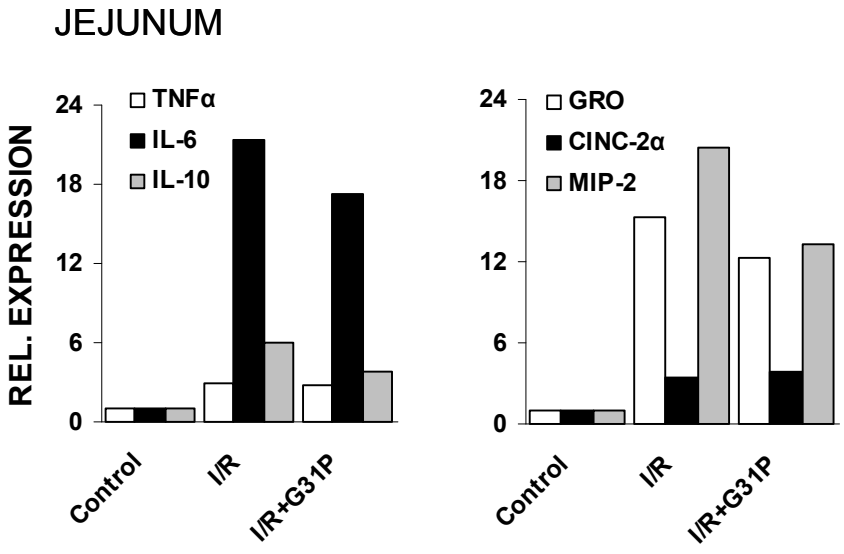


Figure 6.5. Effect of G31P treatments on expression of proinflammatory cytokine and chemokine in I/R injury gut tissues.

Jejunal tissues obtained as in Fig. 6.3 were also processed for total RNA extraction, and the levels of TNFα, IL-β, IL-6, IL-10, GRO, CINC-2α, and MIP-2 expression were assayed by qRT-PCR, as in the *Materials and Methods* section. The data are expressed as the relative quantities of mRNA relative to a calibrator sample (a representative sham surgical group sample). The results indicate that I/R injury differentially increased expression of TNFα, IL-6, IL-10, GRO, MIP-2, and CINC-2 relative to the sham-surgical animals. The G31P treatments can modestly reduced IL-6 and IL-10, as well as GRO expression and more effectively reduced MIP-2 expression. Thes results depicted are from one experiment that is representative of two independent experiments.

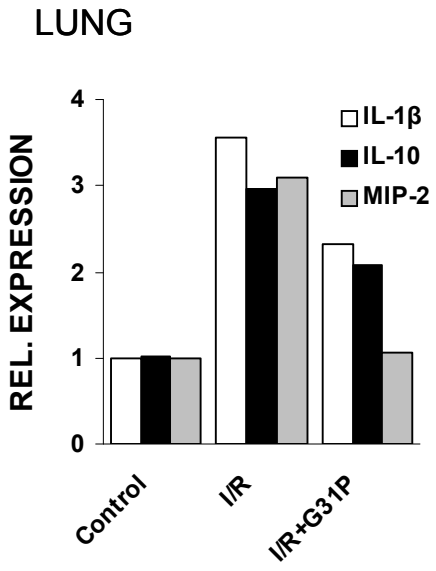


Figure 6.6. Effect of G31P treatments on expression of proinflammatory cytokine and chemokine in I/R injury lung tissues.

Lung tissues obtained as in Fig. 6.1 were also processed for total RNA extraction, and the levels of cytokine and chemokine expression were assayed by qRT-PCR, as in Fig. 5. The data are expressed as the relative quantities of mRNA relative to a calibrator sample (a representative sham surgical group sample). The results indicate that I/R injury differentially increased expression of IL-1β, IL-10, and MIP-2, but not TNF, IL-6, GRO, or CINC-2 (data not shown), relative to the sham-surgical animals. The G31P treatment modestly reduced IL-6 and IL-10 expression, and reduced MIP-2 expression to background. These results are from one experiment that is representative of two independent experiments.

injury (de Vries, Kohl et al. 2003), and that macrophage depletion instead substantially ameliorates such pathology (Chen, Lui et al. 2004). Nevertheless, we observed very significant sparing of local and remote tissue injury by simultaneous blockade of all ELR-CXC chemokines.

We have generated multiple isoforms of the ELR-CXC chemokine antagonist, $CXCL8_{(3-72)}$K11R/G31P (i.e., G31P) and each of these are equally effective in blocking human ELR-CXC chemokine-dependent neutrophil activation (bG31P, hbG31P, hG31P; ref (Li, Zhang et al. 2002; Zhao, Li et al. 2007) and footnote a). *In vitro*, G31P antagonizes CXCR1 and CXCR2 ligand-induced intracellular Ca^{2+} flux, ROI production and chemotaxis in neutrophils, as well as over-riding their anti-apoptotic influence on these cells. But G31P also dampens neutrophil activation (i.e., intracellular Ca^{2+} flux, chemotaxis) induced by ligands for heterologous G protein-coupled receptors (GPCR), including those for LTB4, C5a, and f-Met-Leu-Phe (Zhao, Town et al. 2009). Potentially also of relevance in the present study, G31P mitigates full activation of epithelial cells that are challenged with endotoxin, reducing their secretion of CXCL1, CXCL8, and IL-6, perhaps by interrupting an ELR-CXC chemokine-dependent autologous activation cascade (Zhao, Town et al. 2009). Interestingly, it has been reported that TLR4 is a critical element in I/R injury pathology (Wu, Chen et al. 2007), which raises the question of whether G31P's protective effects could have been in part related to its ability to antagonize endotoxin-induced epithelial cell activation. It is known that epithelial cell apoptosis is prominent in intestinal I/R injury (Coopersmith, O'Donnell et al. 1999) and our histopathology data confirms that the intestinal epithelium suffered catastrophic damage in our I/R injury animals, but that the G31P treatment significantly ameliorated this.

G31P was developed to target neutrophils and, more specifically, their responses to ELR-CXC chemokines. As such, it dampens the pathologic outcomes of the neutrophilic inflammatory responses that are prominent in experimental airway endotoxemia (Gordon, Li et al. 2005), environmental pollutant exposure (Podechard, Lecureur et al. 2008), and aspiration pneumonia (Chapter 5). Herein, we used a rat superior mesenteric artery (SMA) I/R model to examine the impact of ELR-CXC chemokine antagonism on intestinal I/R-induced local and remote organ injury. We found that G31P significantly blocked SMA I/R-induced neutrophil sequestration in the lung and modestly inhibited neutrophil marker

MPO and its pathologic sequellae in the gut. Given that the jejunal tissues were so severely disrupted in our saline-treated I/R injury animals we were not able to assess neutrophil infiltration directly, but our surrogate markers (MPO, MMP-9) clearly suggested that they had been recruited. MPO is routinely used as a marker of neutrophil sequestration (Souza, Bertini et al. 2004), although it would not differentiate between intravascular and extravascular neutrophils. Our data confirms however that ELR-CXC chemokine antagonism had a decided sparing effect on neutrophil recruitment from the vasculature into the lung tissues. Analogous effects on tissue MPO levels have been reported previously with the small molecule allosteric CXCR1/CXCR2 inhibitor, repertaxin (30 mg/kg), although it apparently is not active against alternate GPCR (i.e., LTB4 or fMLP) (Souza, Bertini et al. 2004). We query whether G31P's effectiveness at the doses employed herein (i.e., 500 μg/kg) could be related to its ability to simultaneously block the CXCR1/CXCR2 and heterologous GPCR (Zhao, Town et al. 2009).

Neutrophils have been confirmed to be critically involved in remote organ injury, e.g., lung (Hernandez, Grisham et al. 1987; Chen, Lui et al. 2004), but their role in mediating gut injury in intestinal I/R injury is still uncertain (Hernandez, Grisham et al. 1987; Simpson, Alon et al. 1993; Chen, Lui et al. 2004). We measured the gut tissue levels of the neutrophil tertiary granule marker MMP-9 as well as MMP-2 expression and found that the G31P treatments effectively inhibited both of these. Others have reported high level expression of MMP-9, largely in neutrophils, following intestinal I/R injury (Robinson, Kelly et al. 2008). They concluded that MMP-9 induction contributes importantly to I/R injury pathology. Our histological data indicates that intestinal I/R induced severe intestinal mucosal injury which was significantly attenuated by G31P treatment. This suggests that the neutrophils were causally involved in this injury, perhaps in part through release MMP-9. MMP-2 is a product of both leukocytes and resident cells such as fibroblasts and epithelial cells (Lempinen, Inkinen et al. 2000; Robinson, Kelly et al. 2008). That G31P blocked its expression may provide further (circumstantial) evidence that this antagonist may act on neutrophils and other resident cells in this model system. We have already documented that G31P acts on bronchial epithelial cells, dampening their responses to bacterial endotoxin (Zhao, Town et al. 2009).

Marked expression of the inflammation-associated cytokines TNFα, IL-1β and IL-6 has been reported previously in both local and remote organ tissues during I/R injury (Caty, Guice et al. 1990; Cuzzocrea, De Sarro et al. 1999; Souza, Cassali et al. 2001; Souza, Bertini et al. 2004). We observed little in the way of IL-1 or TNF expression in either the lung or gut in our animals, although IL-6 expression was strongly upregulated in the gut (but not lungs). Given that IL-6 reportedly facilitates TNFα and IL-1β production (Cuzzocrea, De Sarro et al. 1999), it is possible that our 2 h time-frame for reperfusion was too brief to allow significant upregulation of IL-1 and TNF. IL-6 is reportedly integral to loss of gut barrier function after hemorrhagic shock (Yang, Han et al. 2003), and others have reported that anti-CINC antibodies significantly reduce circulating levels of IL-6 in such models (Kaneko, Tamura et al. 2007). Our data confirms that intestinal I/R injury augments IL-6 expression in the gut, and that ELR-CXC chemokine antagonism can effectively decrease this IL-6 expression. This opens up the question of whether G31P's effects in maintaining jejunal integrity were related to its effects on IL-6. Expression of IL-10, an anti-inflammatory cytokine, was increased significantly in the gut and modestly so in the lungs of our I/R injury animals and the G31P treatments had marginal impact on its production in either compartment. It has been reported that blocking the ELR-CXC chemokines by either CXCR1/CXR2 antagonists or with anti-CINC-1 antibodies slightly increases lung, but not the gut, tissue IL-10 expression (Souza, Bertini et al. 2004). However, other studies suggest that IL-10 does not play a significant role in protecting against intestinal I/R injury, but rather that it may exacerbate the tissue injuries (Stallion, Kou et al. 2002; Nussler, Muller et al. 2003).

In conclusion, our results indicate that treatment with G31P, a CXCR1 and CXCR2 antagonist that also dampens responses across heterologous GPCR (i.e., C5aR, BLT1, FPR) on ELR-CXC chemokine receptor-positive cells, has potent effects on local and remote organ tissue neutrophil sequestration, and thereby protects against related tissue injury. This suggests that the approach of broadly blocking the ELR-CXC chemokines may be effective at preventing ischemia and reperfusion injuries in clinical situations.

CHAPTER 7：GENERAL DISCUSSION AND CONCLUSION

Excessive neutrophil recruitment and activation have been considered a hallmark of acute lung injury (ALI) and the more severe form of disease, acute respiratory distress syndrome (ARDS). These two disorders are responsible for 40-60% of mortality in clinical intensive care units (Repine and Beehler 1991; Ware and Matthay 2000; Martin 2002). ALI/ARDS can be caused by a variety of direct (e.g., aspiration of gastric content) and indirect (e.g., ischemia and reperfusion) insults (Bernard, Luce et al. 1987; Gerkin, Oldham et al. 1993). ELR-CXC chemokines (e.g., CXCL8) play a pivotal role in these situations so that their blockade significantly attenuates pathology and mortality (Shanley, Davidson et al. 2000; Souza, Bertini et al. 2004). Although there are a large number of ELR-CXC chemokines, there are only two ELR-CXC chemokine receptors, CXCR1 and CXCR2. It is understandable that blockade of these receptors would be a more efficient approach than the blockade of any single chemokine. It is also known that CXCL8 binds these two receptors with higher affinity than other members of this chemokine family (Mukaida 2003) so that CXCL8 was an ideal candidate to be developed into a competitive CXCR1 and CXCR2 antagonist.

The purpose of this research project was to develop a humanized or human form of the ELR-CXC chemokine antagonist, bovine $CXCL8_{(3–74)}$K11R/G31P (bG31P). bG31P was previously developed by a postdoctoral fellow (Dr. Fang Li) in our lab. Her data have substantially demonstrated that bG31P is a very high affinity broad-spectrum ELR-CXC chemokine antagonist (Li and Gordon 2002; Li, Zhang et al. 2002). This antagonist effectively blocked CXCL8 binding to, and its activation of, neutrophils *in vitro*. It fully antagonized pulmonary neutrophil responses to intradermal or airway endotoxin challenge of cattle or guinea pigs, respectively. It also blocked pyrexia and pleural hemorrhagic consolidation in endotoxin-challenged animals (Li, Zhang et al. 2002; Gordon, Li et al. 2005). While bG31P could perhaps be useful in the humans, it goes without saying that a human equivalent thereof would be of significantly more use.

Prior structure-function studies have indicated that human CXCL8$_{(3-72)}$K11R is not a particularly high affinity neutrophil agonist, and that a combined G31P/P32G substitution within human CXCL8$_{(4-72)}$ only marginally reduces its neutrophil agonist activities (Clark-Lewis, Dewald et al. 1994), suggesting that human CXCL8$_{(3-72)}$K11R/G31P (i.e., the human counterpart of bG31P) would perhaps not be an effective chemokine antagonist as discussed above (Chapter 2 rational). To develop a human form of this bovine ELR-CXC chemokine antagonist, my study began with the humanization of bG31P. A human-bovine chimeric protein, bovine CXCL8$_{(3-44)}$K11R/G31P-hCXC8$_{(45-72)}$ (i.e., hbG31P), was generated and its antagonist activities were characterized. To further humanize hbG31P, one-by-one we made substitutions of the remaining amino acids that were discrepant between human and bovine CXCL8 (i.e., T3/K, H13/Y, H15/K, E35/A, and S37/T). The antagonist activities of these new molecules were also assessed. As shown in Chapter 3, hbG31P fully retained the antagonist activity of bG31P for human neutrophils and its further humanized analogues did not show significantly better blocking activity.

Based on these findings, we began to characterize a fully human form of bG31P, human CXCL8 $_{(3-72)}$K11R/G31P (hG31P) that was generated in our laboratory. hG31P was also tested in guinea pig models of airway endotoxemia and aspiration pneumonia, as well as a rat model of intestinal ischemia and reperfusion injury. As shown in Chapters 4, 5, and 6, hG31P potently blocks ELR-CXC chemokine-induced neutrophil activation. The anti-inflammatory effects of hG31P extended to reversing ELR-CXC chemokine-induced delays in neutrophil apoptosis, desensitizing heterologous GPCRs and inhibiting endotoxin-induced chemokine expression by epithelial cells. In *in vivo* systems, hG31P was highly effective in blocking the pathology associated with airway endotoxemia, aspiration pneumonia, and intestinal ischemia and reperfusion injury, including neutrophil recruitment into, and activation within, the airways or gut, chemokine or cytokine expression, and pulmonary vascular complications. The blockade of neutrophil recruitment by hG31P in aspiration pneumonia animals did not foster airway bacterial outgrowth. hG31P treatment was also protective in both mesenteric artery I/R-induced local and romote organ injury.

To summarize the findings of this thesis, it is clear that:

1. The chimeric protein hbG31P and its analogues effectively block chemotactic responses, ROI release, and calcium infux of human neutrophils stimulated by human ELR-CXC chemokines *in vitro*. hbG31P has also been shown to have potent antagonist activity in blocking pulmonary neutrophil infiltration, as well as the loss of vascular integrity in endotoxin-induced acute lung injury in a standard guinea pig endotoxemia model. hbG31P fully retains the antagonist activity of bG31P.

2. A fully human form of bG31P, $CXCL8_{(3-72)}$K11R/G31P (hG31P), was successfully generated. hG31P was shown to be a CXCR1/CXCR2 antagonist by its abilities to inhibit IL-8-induced chemotactic responses and intracellular Ca^{2+} mobilization in CXCR1-transfected HEK cells and human neutrophils, and responses induced by CXCR2-exclusive ligands. hG31P also desensitized heterologous GPCRs (e.g., BLT, C5aR, and FPR) on neutrophils and blocked LPS-induced ELR-CXC chemokine (e.g., CXCL1, CXCL8) production by bronchial epithelial cells. The anti-inflammatory effects of hG31P extended also to reversing the anti-apoptotic influence of ELR-CXC chemokines on neutrophils. In a guinea pig model of airway endotoxemia, hG31P also effectively blocked neutrophil infiltration into, and activation within, the airways. We conclude that the beneficial effects of ELR-CXC chemokine receptor blockade by hG31P treatment occur at multiple levels, including epithelial cells, neutrophils, and alternate GPCRs.

3. When hG31P was applied in an aspiration pneumonia model, the treatment dramatically attenuated the pulmonary vascular complications and pulmonary neutrophilia, and reduced airway levels of neutrophil degranulation products. The blocking effect of hG31P also extended to reducing lung tissue CXCL1 and CXCL8 expression. This attenuation of the neutrophil response was not associated with rampant airway bacteria outgrowth at 20 h after gastric content challenge. Therefore, these data suggest that alternate bacterial clearance mechanisms may operate efficiently in the absence of this overt pathology or at least efficiently enough to hold bacteria growth in check.

4. The antagonist activities of hG31P treatment were also demonstrated in a rat superior mesenteric artery (SMA) I/R injury model. hG31P significantly inhibited neutrophil infiltration into the lung as well as pulmonary vascular leakage, and significantly decreased neutrophil margination in the circulation of I/R challenged animals. The hG31P treatment also partially reduced the loss of mucosal integrity and proteinase expression within the jejunum. Moreover, hG31P treatment blocked intestinal I/R-induced local and remote organ expression of inflammatory cytokines and ELR-CXC chemokines. These findings suggest that ELR-CXC chemokine antagonism has significant protective effects against I/R-induced local and remote organ injury.

In general, these findings suggest that G31P (hereafter, all types of G31P): can significantly blunt neutrophil activation (e.g., calcium flux, ROI release, degranulation) and recruitment; can effectively ameliorate animal pyrexia and vasculature leakage; and also can dramatically decrease tissue chemokine (e.g., CXCL1, -8, MIP-2) and cytokine (e.g., IL-6) expression.

As reviewed in Chapter 1 (§1.2.3.4.), CXCL8 binds the CXCR1 or CXCR2 on neutrophils via two major sites, the ELR (Glu^4-Leu^5-Arg^6) motif preceding the first cysteine and the receptor recognition loop region (residues 10-17) between the second and the third cysteines (the Gly^{31}-Pro^{32} motif). The loop region functions as a docking domain which determines the chemokine's binding affinities (Baggiolini 2001). During the process of developing G31P, our intitial truncation of first two amino acids and substitution of Lys11 (within the loop region) of CXCL8 with an Arg residue dramatically increased its (i.e., $bCXCL8_{(3\text{-}74)}K11R$) binding affinity (Li and Gordon 2001). Further substitution of Gly31 with a Pro residue turned $CXCL8_{(3\text{-}72)}K11R$ into a potent antagonist (Li and Gordon 2002). Recent studies by David Cheng group indicated that the substitution of Gly^{31} with Pro changes the three-dimensional structure of CXCL8 by affecting the Cys^7-Cys^{34} disulfide bridge, resulting in a conformational change to the ELR motif (Clark-Lewis, Dewald et al. 1994) and thereby dampening ELR motif signaling.

Prior to developing a human form of bG31P, we found that $CXCL8_{(3\text{-}72)}K11R$ does not have a much higher binding affinity than wild type CXCL8 (Clark Lewis, Dewald et al.

1994). We also found that $CXCL8_{(4-72)}$G31P/P32G has much lower binding affinity than wild type CXCL8 (Clark Lewis, Dewald et al. 1994), which raised the question of whether human $CXCL8_{(3-72)}$K11R/G31P could be a practical antagonist clinically. Thus we generated a human-bovine chimeric ELR-CXC chemokine antagonist, i.e. hbG31P, but also a fully human G31P. I undertook the humanization of bG31P, others generated hG31P. Despite our supposition, the data from hbG31P and its analogues suggested that fully human form of bG31P might be a functional antagonist (Zhao, Li et al. 2007), and indeed we demonstrated that hG31P is a potent ELR-CXC chemokine antagonist. Why hG31P turned to be an antagonist could potentially be because the substitutation of Gly for Pro31 affects the scaffold structure of CXCL8, particularly when this was in concert with the Arg substitution for Lys. The effect of this substitution could differentially affects $CXCL8_{(4-72)}$ and $CXCL8_{(3-72)}$.

As hG31P was shown to be effective in blocking several important inflammatory responses, there is substantial interest in understanding how it does this. The most impressive finding of this thesis is the potent effects of G31P on blocking neutrophil infiltration into, and activation within, the inflamed tissues. Neutrophil migration and activation can be attributed to several groups of important inflammatory mediators, such as proinflammatory cytokines (e.g., TNFα, IL-1), chemoattractants (e.g., C5a, LTB4, PAF, ELR-CXC chemokines, fMLP), and adhesion molecules (e.g., β2-integrin, P-, L-selectin, ICAM-1). These molecules are found within or expressed by plasma (e.g., C5a), leukocytes (e.g., monocytes, neutrophils), and resident cells (e.g., endothelial cells, epithelial cells, macrophages).

G31P may directly block neutrophil activation induced by proinflammatory mediators (e.g., ELR-CXC chemokines, C5a). We knew that G31P blocks ELR-CXC chemokine-induced neutrophil activation. We had shown that bG31P has higher affinity for neutrophils than CXCL8 and binds via both the CXCR1 and CXCR2 (Li and Gordon 2002). Given that CXCL8 has higher affinity for these receptors than their other ligands (e.g., CXCL1, CXCL5), it is easy to understand that G31P also has a higher binding affinity for the CXCR1 and CXCR2 than other ELR-CXC chemokines. G31P, therefore, can competitively bind both receptors and block ELR-CXC chemokine binding and thereby inhibit their agonist activities. We propose that this process occurs when the

neutrophils are still in the blood stream. In *ex vivo* experiments, our results showed that circulating neutrophils from hG31P-treated and gastric content-challenged guinea pigs were hyporesponsive to CXCL8-induced chemotactic responses (Chapter 5). Our lab also has previously shown similar results when using bG31P treatment in airway endotoxemic animals (Gordon, Li et al. 2005). This desensitization of neutrophil responses to environmental ELR-CXC chemokines may also happen in the tissue. When using neutrophil chemotaxis assays, we have also confirmed that bG31P strongly antagonizes neutrophil responses to CXCL8 (Li and Gordon 2002). In our recent studies, hbG31P and hG31P were also shown to effectively block neutrophil chemotactic responses to CXCL8, CXCL1, and CXCL5 (Chapters 3 and 4). We also found that hbG31P and hG31P can inhibit CXCL8-induced white blood cell ROI release and CXCL8- and CXCL1-induced neutrophil calcium mobilization. Taken together, G31P can competitively bind both the CXCR1 and CXCR2 receptors and can effectively antagonize ELR-CXC chemokine-induced neutrophil activation and chemotactic responses.

Secondly, through binding the CXCR1 and CXCR2, G31P may desensitize the alternate receptors on neutrophils for other chemoattractants and prevent excessive neutrophil activation by these molecules. Some studies showed that CXCL8 can heterologously desensitize neutrophil receptors for fMLP, C5a, LTB4 and PAF through binding the CXCR1 (Richardson, Pridgen et al. 1998; Ali, Richardson et al. 1999). Given that G31P has a higher affinity for the CXCR1 and CXCR2 than wild type CXCL8, it is reasonable that G31P also would desensitize neutrophil receptors for fMLP, C5a, LTB4, and PAF. Our results in Chapter 4 confirmed that G31P inhibits fMLP-, C5a-, and LTB4-induced neutrophil chemotactic and calcium flux responses, which suggests that G31P may heterologously desensitize their respective GPCRs. Although we did not test if G31P can block PAF-induced neutrophil activation, the heterologous desensitization effect of G31P may also extend to the PAF receptor inasmuch as CXCL8 can do so. Based on these findings, we conclude that G31P can also block fMLP-, C5a-, LTB4-, and PAF-induced neutrophil activation and chemotactic responses. In summary, G31P may block both ELR-CXC chemokines- and other neutrophil chemoattractant-induced neutrophil migration and activation, which could explain why G31P has such potent effects on blocking neutrophils recruitment into and activation within the airway.

Besides the blockade of neutrophil calcium mobilization and ROI release, G31P may also block other aspects of neutrophil activation, such as degranulation, integrin activation and L-selectin shedding. bG31P was shown to effectively inhibit CXCL8-induced β-glucuronidase (neutrophil primary granule) release (Li and Gordon 2002). Other study also showed that an anti-CXCR2 antibody or nonpeptide CXCR2 antagonist can effectively block IL-8-induced MMP-9 (neutrophil tertiary granule) release (Chakrabarti and Patel 2005). In our *in vivo* systems, we have demonstrated that hG31P treatment can dramatically reduce the levels of neutrophil granule markers MPO, lactoferrin, and MMP-9 production in the airway or lung tissue, which may thereby reduce the granule enzyme-induced tissue damage. As MMP-9 can also facilitate neutrophil transendothelial cell migration through its collagenase activities, the reduction of the release of this enzyme may contribute to the blockade of neutrophil recruitment. Our results did not provide direct evidence that G31P can affect expression of integrin and L-selectin on neutrophils, but ELR-CXC chemokines have been shown to induce neutrophil β2-integrin (Mac-1) up-regulation and L-selectin shedding (Bajt, Farhood et al. 2001). As expression of these two molecules is dependent on neutrophil calcium mobilization, and as hG31P blocks CXCL1- and CXCL8-induced neutrophil calcium flux, it seems likely that it also decreases the expression of adhesion molecules, resulting in decreased neutrophil transmigration. In addition to the inhibition of excessive neutrophil activation, our data indicate that G31P also can reverse CXCL1- and CXCL8-induced anti-apoptotic effects on neutrophils and thereby inappropriate prolongation of their life-span in the circulation or tissue, which in turn may reduce the tissue damage brought by activated neutrophils. The mechanisms involved in G31P-induced blockade of neutrophil activation will be discussed below.

Given that CXCL8 can homologously desensitize CXCR1 and CXCR2 responses to CXCL8 and other ELR-CXC chemokines, and that G31P has a higher binding affinity, the reason that G31P can effectively block ELR-CXC chemokine-induced neutrophil activation may be attributed to a higher ability for homologous desensitization of CXCR1 and CXCR2. CXCL8- or G31P-induced receptors homologous desensitization is mainly caused by CXCR1 and CXCR2 phosphorylation and internalization. Sustained G31P-induced CXCR1 and CXCR2 desensitization may lead to the blockade of neutrophil

activation by wild type ELR-CXC chemokines. As discussed above, CXCL8 is also able to heterologously desensitize the GPCRs for C5a, fMLP, LTB4 and PAF. The ability of CXCL8 to desensitize the C5a and fMLP receptors is more limited than those of C5a and fMLP to desensitize CXCR1 (Richardson, Pridgen et al. 1998; Ali, Richardson et al. 1999). The reason is because CXCL8 signals neutrophils mainly through the PI3K/Akt pathway, whereas fMLP and C5a mainly use the p38MAPK-dependent pathway. In inflammatory settings, when facing different signals, neutrophils adopt hierarchical responses to fMLP and C5a, which activate MAPK, downstream signaling of which dampens PI3K/Akt signaling. CXCL8 was also shown to heterologously desensitize fMLP and C5a receptors, which may be because CXCL8-stimulated PI3K/Akt signaling is reciprocally able to dampen MAPK signaling, but at reduced levels, or it may activate MAPK signaling cause MAP kinase phosphorylation and thereby reduced responsive to C5a or fMLP stimulation. Upon CXCL8 binding, heterotrimeric G-protein can be activated by the exchange of GDP for GTP, which disassociates into GTP-bound α and βγ subunits. The βγ subunit, which can activate PI3K signaling pathway, may also activate MAPK signaling (Baggiolini 2001; Thelen 2001). CXCL8 provides weaker signals for heterologous desensitization than fMLP and C5a possibly because of less resistance of phosphorylation of CXCR1 and CXCR2 than FPR and C5aR do (Ali, Richardson et al. 1999). The reasons that G31P improved the strength of heterologous desensitization compared with CXCL8 may be that, firstly, its high affinity binding to the receptors possibly strengthens the interference of PI3K signaling with MAPK signaling or directly increases stronger phosphorylation of MAPK and thereby leads to reduced neutrophil responses to fMLP and C5a. Secondly, the CXCR1 and CXCR2 may become more resistant to phosphorylation induced by fMLP and C5a after higher affinity binding by G31P.

The airway epithelium, as a primary interface between the host and the environment, plays a crucial role in the innate immune response (Becker, Diamond et al. 2000). We have evidence that G31P treatment can decrease tissue cytokine and chemokine expression *in vivo*. We also showed that hG31P dose-dependently decreases LPS-induced cytokine (e.g., IL-6) and chemokine (e.g., CXCL1, CXCL8) expression by pulmonary epithelial cells. We speculate that G31P may indirectly block epithelial cell activation induced by LPS through interrupting LPS-TLR4 signaling (e.g., MAPK, ERK

phosphorylation) through its activated PI3K signaling pathway, which is similar to heterologues desensitization of other GPCR, and it may also directly block ELR-CXC chemokines (e.g., CXCL8)-induced epithelial cell activation. Pulmonary epithelial cells may express CXCR2, which is found to be important in neutrophil recruitment and increased pulmonary permeability in LPS-induced acute lung injury (Reutershan, Morris et al. 2006). Since epithelial cells are the important sources of CXCL8 (Fujii, Hayashi et al. 2002; Sha, Truong-Tran et al. 2004), which may bind their CXCR2 in an autocrine fashion and further activate these cells, CXCL8 production in the airway presumably forms part of an autocrine system. G31P, as a CXCR1 and CXCR2 antagonist, may block CXCL8-induced epithelial cell activation, reducing its own expression. The LPS-TLR4 signaling pathway also mediates LPS-induced epithelial cell activation, which involves a signaling complex including the kinases interleukin-1 receptor-associated kinase, p38, Jnk, and ERK1/2 (Guillot, Medjane et al. 2004). The results from a postdoctoral fellow (Dr. Wenjun Wang) in our lab have indicated that G31P blocks CXCL8-induced ERK1/2 phosphorylation in neutrophils (unpublished data), which suggest that G31P may also be able to block the ERK1/2 phosphorylation in epithelial cells and reduce their activation (e.g., cytokine and chemokine expression).

Our results also showed that G31P dramatically reduced the release of inflammatory mediators (e.g., IL-6, IL-8) during neutrophil-epithelial cell interactions. We only examined IL-6 and IL-8 expression in the neutrophil and A549 epithelial cell co-cultures, and do not know which population of cells was activated and therefore responsible for the cytokine and chemokine release. Some studies have demonstrated that neutrophil elastase played an important role in inducing airway epithelial cells to produce IL-8 (Devaney, Greene et al. 2003; Chen, Lin et al. 2004). Neutrophil defensins and TNFα also can induce a dramatic level of IL-8 and ENA-78 expression from alveolar epithelial cells (e.g., A549 cell) and increase expression of IL-8 and IL-6 from bronchial epithelial cells (van Wetering, Mannesse-Lazeroms et al. 2002). Epithelial-derived IL-8 and ENA-78 can further activate neutrophils to degranulate and release more pro-inflammatory mediators (e.g., IL-1β, TNFα) and therefore amplify these reciprocal inflammatory responses. Neutrophil elastase can also induce airway epithelial cell apoptosis, which may lead to epithelial barrier disruption, increasing alveolar epithelial permeability and thereby

causing alveolar edema (Moraes, Zurawska et al. 2006). G31P can inhibit further neutrophil activation by blocking chemokine signaling or may also block the activation of epithelial cells and reduce further IL-8 production. Either way of G31P does interrupt these intercellular interactions sufficiently to ameliorate their mutually expanding inflammatory responses.

As examined in the literature review (§1.2.2.1.2.), vascular endothelial cells play important roles in mediating neutrophil migration during neutrophilic inflammation. Once activated by bacterial products (e.g., LPS), proinflammatory cytokines (e.g., TNFα, IL-1β), or chemoattractants (e.g., C5a, IL-8, MIP-2), they can express large amounts of selectins (P-selectin, E-selectin), proinflammatory cytokines (e.g., TNF, IL-1) and chemoattractans (e.g., PAF, IL-8) (Nawroth, Bank et al. 1986; Grace 1994; Ranta, Orpana et al. 1999). These inflammatory mediators are essential in the process of neutrophil transendothelial migration (Smart and Casale 1994), but they also amplify the inflammatory responses, resulting in endothelial cell damage and disruption of vascular integrity (Mantovani, Bussolino et al. 1992). In LPS-induced animal airway endotoxemia, we found dramatic areas of hemorrhagic consolidation on the pleural surface and substantial RBC extravasation into the airways. G31P treatments very significantly ameliorated these two pathologic features. As mentioned above, neutrophil transendothelial migration contributes significantly to vascular endothelial damage and the increased vascular permeability in a variety of ways. In the case of LPS-induced acute lung injury, LPS can stimulate endothelial cells and other resident cells to release TNFα and IL-1β, which can induce the release of vasodilatory factor, PGI_2, leading to increasing vascular permeability (Mantovani, Bussolino et al. 1992). These two molecules can also stimulate neutrophils and epithelial cells to produce MMP-9, which can degrade basement membrane constituents of the endothelium, resulting in endothelial damage (Corbel, Boichot et al. 2000). TNFα and IL-1β may directly induce endothelial injury by the end-products (HO*) of the Fenton's (oxidization) reaction and/or the generation of prostanoids in endothelial cells, which is not yet fully understood (Lentsch and Ward 2000). Neutrophil-derived elastase can facilitate endothelial cell ROI generation and release and further induce endothelial cytotoxity. Neutrophil-derived ROI can induce phosphorylation of focal adhesion kinase in endothelial cells, resulting in disruption of intercellular tight junctions,

redistribution of focal adhesions, and thereby cause vasulature damage (Usatyuk and Natarajan 2005). Thus, the endothelial cell damage induced by activated neutrophils requires products from both the neutrophil (e.g., ROI, elastase, MMP-9) and the endothelial cell (e.g., ROI) (Lentsch and Ward 2000). In summary, all the damage to vascular endothelial cells, basement membrane, and matrix components induced by the above-mentioned mediators during neutrophil and endothelial cell interaction can lead to loss of pulmonary vascular integrity and result in pulmonary edema and microvascular haemorrhage. The effects of G31P on blocking neutrophil activation have been discussed above. Next, the possible roles of G31P in inhibiting endothelial cell activation will be discussed below.

Similar to neutrophils, ELR-CXC chemokines activate endothelial cells through binding to the CXCR1 and CXCR2, which are expressed in human umbilical vein endothelial cells (HUVEC) and human lung microvascular endothelial cells (HMVEC). IL-8 leads to endothelial cell cytoskeletal reorganization, which may contribute to the increased vascular permeability (Schraufstatter, Chung et al. 2001). This notion was confirmed in a LPS-induced acute lung injury model (Reutershan, Morris et al. 2006). The effect of G31P on CXCR2 signaling may ameliorate the vascular permeability changes and hemorrhage induced by LPS challenge in the lung tissue. ELR-CXC chemokines (e.g., MIP-2, KC) also play important roles in mediating P-selectin expression on endothelial cells, presumably also through CXCR2 signaling (Zhang, Liu et al. 2001). As reviewed in Chapter 1, P-selectin is one of the major selectins mediating neutrophil rolling. Blocking of P-selectin expression can inhibit neutrophil rolling (Johnston, Walter et al. 1997). G31P might play an important role in blocking ELR-CXC chemokine-induced P-selectin expression therefore preventing neutrophil migration. Studies have also found that IL-6 plays critical roles in increasing endothelial permeability during tissue hypoxia and I/R injury (Ali, Schlidt et al. 1999; Klein, Hoke et al. 2008). The effect of G31P on IL-6 production during neutrophil-epithelial cell interactions or by LPS-induced epithelial cells may contribute to the reduction of lung permeability changes. In our intestinal I/R model, we had evidence that G31P treatment can dramatically reduce IL-6 expression in lung and gut tissues. In conclusion, neutrophil transendothelial migration activates endothelial cells and cause vascular damage and cytokines, chemokines, and adhesion molecules play

important roles in this process. The antagonist activities of G31P in blocking the expression these molecules can ameliorate vascular damage.

G31P treatment was also demonstrated in our aspiration pneumonia and I/R injury models to effectively reduce chemokine (e.g., IL-8, MIP-2) and cytokine (e.g., TNF, IL-1, IL-6) responses. Epithelial cells are known to be a primary source of cytokines and chemokines in inflammatory settings. As discussed above, firstly, our results have documented that G31P can significantly inhibit IL-8 and IL-6 release from LPS-challenged epithelial cells and during neutrophil-epithelial cell interactions. Its blockade of neutrophil activation may also inhibit the expression of other chemokines, such as ENA-78, because neutrophil defensins can induce ENA-78 production from epithelial cells (van Wetering, Mannesse-Lazeroms et al. 2002). In endothelial-epithelial cell co-cultures, the endothelial cells stimulated the epithelial cells to release IL-6 and reciprocally, the epithelial cells enhanced the release of IL-8 from the endothelial cell, which facilitates neutrophil transmigration (Mul, Zuurbier et al. 2000). G31P blockade of neutrophil activation, particularly CXCR1 or CXCR2 signaling, may reduce this reciprocal effect and decrease cytokine and chemokine expression. As reviewed in Chapter 1 (§ 1.2.1.4), LPS induces macrophages/monocytes, neutrophils, endothelial cells and epithelial cells to produce proinflammatory cytokines, ELR-CXC chemokines and adhesion molecules. We have evidence that G31P blocks LPS-induced cytokine and chemokine expression from epithelial cells, but we do not have direct evidences on its effects on other cell types. It is not surprising that G31P would reduce LPS-induced neutrophil and endothelial cell activation as their expression of CXCR1 and CXCR2 is critical in their activation. But further studies are required to confirm these proposals.

To give an overview of G31P therapeutic functions, I summarized the possible mechanisms of G31P in blocking neutrophil or other resident cell activation in airway enodoxemia and intestinal I/R injury in figures 7.1. and 7.2..

Our results suggest that G31P-mediated inhibition of neutrophil recruitment does not dampen bacterial clearance in the airway, which was assessed at 20 h after gastric content challenge. As neutrophils are major effector cells that phagocytose foreign pathogens, we would need to examine bacterial outgrowth after 96 h or more to see if

neutrophil blockade by G31P affects longer term outcomes. It is also worth determining if the functions of other cell types (e.g., macrophages/monocytes) are changed or if alternate defense weaponary (e.g., epithelial defensins) are functionally increased or otherwise altered.

We evaluated the antagonist activities of G31P in several model systems, including airway endotoxemia, aspiration pneumonia, and mesenteric I/R injury. It seems that the effects of G31P on neutrophil infiltration into the gut and its amelioration of gut pathology in the I/R injury model were somehow less pronounced than its effects in the other two models. The question raised here is in which kinds of diseases G31P can be effective and via which route it should be administered. Our lab has previously demonstrated that G31P given intramuscularly (i.m.) and subcutaneously (s.c.) is more effective than when given i.v. in dermal LPS challenge reactions (Li, Zhang et al. 2002). In mesenteric I/R injury, G31P was shown to ameliorate neutrophilic pathology but not as profoundly as during aspiration pneumonia or airway endotoxemia, when given by the same route. Other ELR-CXC chemokine antagonists, such as repertaxin, have been given i.v. to I/R injury animals. (Souza, Bertini et al. 2004). When it was used in a rat liver I/R injury model (1 h of ischaemia and 12 h of reperfusion) and given via both s.c. and i.v., before and after reperfusion, respectively, it was effective in blocking neutrophil infiltration into the liver. In the same report, in a cecal ligation and puncture (CLP) model, when it is given s.c multiple times (e.g., before and after the CLP), it significantly reduced neutrophil infiltration into the peritoneal cavity (Bertini, Allegretti et al. 2004). Another drug, $GRO\alpha_{(8-73)}$ (i.e., GROα without a complete ELR motif, which thereafter functions as aPMN antagonist), was given via i.p. in a peritonitis model and also shown to effectively block neutrophil infiltration (McColl and Clark Lewis 1999). All of these suggest that G31P could perhaps also be effective in blocking neutrophilic pathology when given via i.v. or i.p. in these settings. The reason may be that i.v. or i.p. delivery may help the drug work at the inflammation site, such as the ischemic organ, and prevent the exacerbation of inflammatory responses. Considering that the I/R injury occurs in a matter of several hours, G31P given via these two ways may be more effective.

Figure 7.1. The effects of G31P in blocking neutrophilia pathology in airway endotoxemia.

During airway endoxemia, LPS can bind to LPS-binding proteins (LBP) and form a LPS-LBP complex, which interacts with CD14 and later with TLR4 on airway macrophages (AM), leading to activation via TLR4 signaling. LPS-TLR4 signaling and C5a (e.g., generated by LPS-induced alternative pathway) can activate AM to produce ELR-CXC chemokines (e.g., CXCL8/IL-8) and proinflammatory cytokines (TNF, IL-1, and IL-6). LPS-TLR4 signaling can also activate airway epithelial cells to release the aforementioned chemokines and cytokines. Cytokines by themselves can further induce chemokine expression by epithelial cells. As airway epithelial cells express CXCR1, ELR-CXC chemokines may bind these receptors inducing further activation and IL-8 expression, and presumably forming an autocrine system. LPS-TLR4 signaling, C5a, and cytokines can also stimulate vascular endothelial cells to produce IL-8 and PAF, which are immobilized on the endothelial cell surface via binding to glycosminoglycans (GAG). The chemokines and other chemoattractants (e.g., PAF and LTB4) can bind to CXCR-1 and -2 or their relative GPCRs (e.g., PAFR, BLT) on neutrophils. This activates β2 integrin and L-selectin expression, degranulation and ROI release, which facilitates neutrophil transendothelial migration and results in vascular damage. Our findings suggest that G31P has anti-inflammatory activities at multiple levels. (1) In circulation, G31P binding CXCR-1 and -2 on neutrophils can prevent ELR-CXC chemokine activation and dampen activation of other chemoattractants through desensitization of their relative GPCRs, which plays a major role in preventing neutrophil transendothelial migration and reducing the vascular damage. (2) G31P may also block neutrophil chemotaxis, ROI release, Ca^{2+} flux and reverse anti-apoptotic effects induced by ELR-CXC chemokines or other chemoattractants (e.g., C5a, LTB4) during their transendothelial migration and migration into the interstitial tissue. (3) G31P can also block LPS-induced ELR-CXC chemokine and cytokine expression by epithelial cells, presumably via breaking chemokine autocrine systems; however this needs to be investigated further. (4) G31P may also block endothelial cell activation and the expression of adhesion molecules, chemokines stimulated by ELR-CXC chemokines, LPS, or other chemoattractants. Whether or not endothelial cells are affected and by what mechanisms is an interesting area for future study.

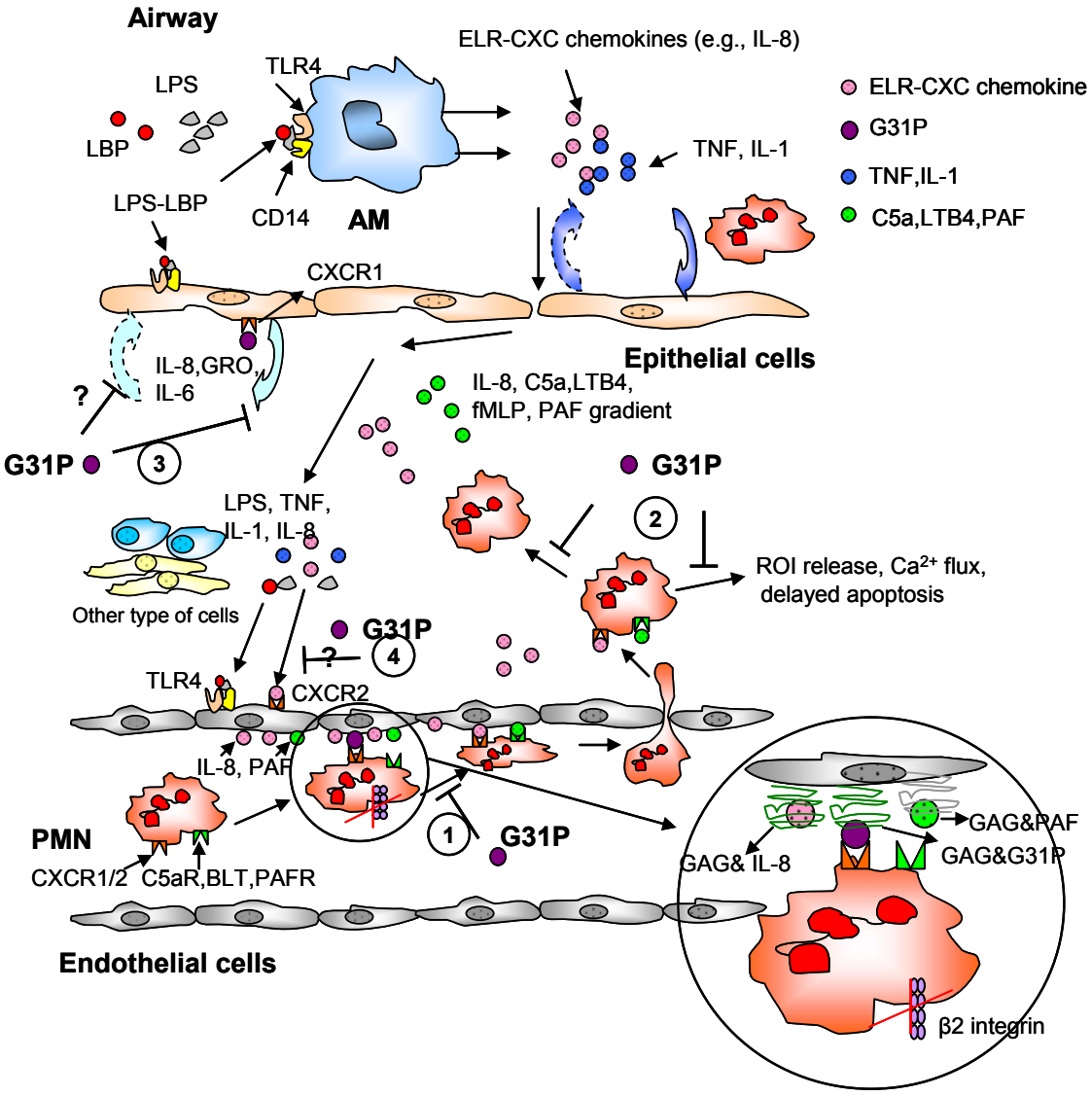
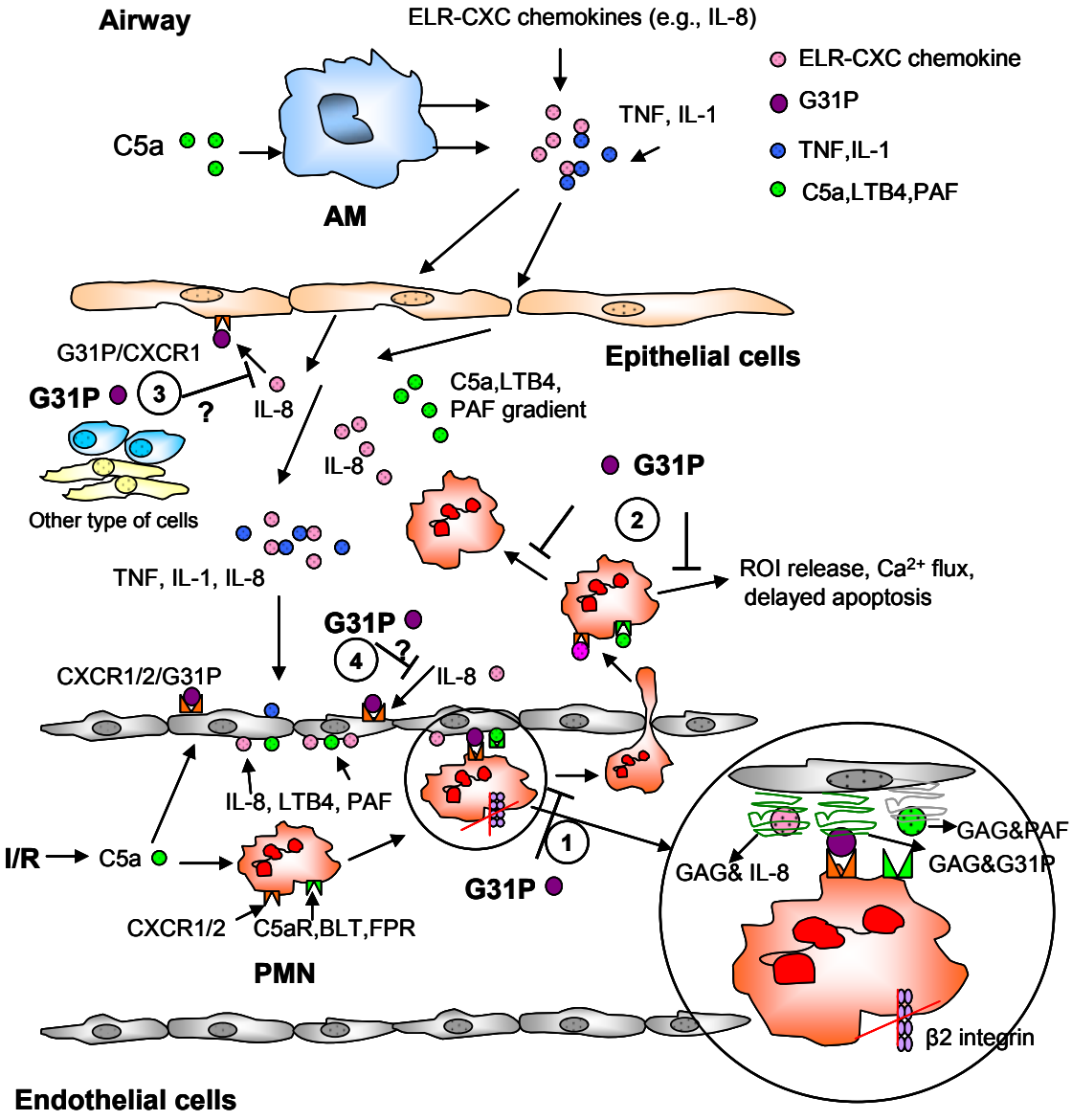

Airway
ELR-CXC chemokines (e.g., IL-8)
TLR4
LPS
LBP
LPS-LBP
CD14
AM
TNF, IL-1
ELR-CXC chemokine
G31P
TNF,IL-1
C5a,LTB4,PAF
CXCR1
Epithelial cells
IL-8,GRO,
IL-6
?
G31P
3
IL-8, C5a,LTB4,
fMLP, PAF gradient
G31P
LPS, TNF,
IL-1, IL-8
2
ROI release, Ca2+ flux,
delayed apoptosis
Other type of cells
G31P
?
4
TLR4
CXCR2
IL-8, PAF
PMN
CXCR1/2
C5aR,BLT,PAFR
1
G31P
GAG&PAF
GAG&G31P
GAG& IL-8
β2 integrin
Endothelial cells

Figure 7.2. The effects of G31P in blocking neutrophilia pathology in intestinal I/R injury.

Hypoxia during intestinal I/R injury can induce endothelial cell necrosis, which in turn initiates complement (e.g., C5a) production mainly via alternative pathways in the circulation and interstitial tissue (Markiewski and Lambris 2007). C5a can bind its receptors, C5aR or C5L2, on the surface of endothelial cells and stimulate IL-8 and expression other chemoattractants (e.g., PAF, LTB4). C5a can activate residential AM to produce ELR-CXC chemokines (e.g., CXCL8/IL-8) and proinflammatory cytokines (TNF, IL-1, and IL-6), which can further activate airway epithelial cells to release the aforementioned chemokines and cytokines. Cytokines by themselves can also induce chemokine expression by epithelial cells. As airway epithelial cells express CXCR1, ELR-CXC chemokines may bind it and further activate epithelial cells to induce IL-8 expression and presumably form an autocrine system. The proinflammatory cytokines can also stimulate vascular endothelial cells to produce IL-8 and PAF, which are immobilized on the endothelial cell surface via binding to glycosminoglycans (GAG). The chemokines and other chemoattractants (e.g., PAF and LTB4) can bind to CXCR-1 and -2 or their relative GPCRs (e.g., PAFR, BLT) on the neutrophils. This activates β2 integrin and L-selectin expression, degranulation and ROI release, which facilitates neutrophil transendothelial migration and results in vascular damage. Similarly, G31P may have the following anti-inflammatory activties. (1) In circulation, G31P binding of CXCR1 and CXCR2 on neutrophils can prevent ELR-CXC chemokine activation and dampen activation and expression of other chemoattractants through desensitization of their relative GPCRs. This prevents neutrophil transendothelial migration and reduces vascular damage. (2) G31P may also block neutrophil chemotaxis, ROI release, Ca^{2+} flux and reverse anti-apoptotic effects induced by ELR-CXC chemokines or other chemoattractants (e.g., C5a, LTB4) during their transendothelial migration and migration in the interstitial tissue. (3) G31P may block ELR-CXC chemokine stimulated epithelial cell activation, presumably via breaking chemokine autocrine systems, however this needs further investigation. (4) G31P may also block endothelial cell activation and the expression of adhesion molecules, chemokines stimulated by ELR-CXC chemokines, LPS, or other chemoattractants. Whether or not endothelial cells are affected and by what mechanisms is an interesting area for future study.


Airway
ELR-CXC chemokines (e.g., IL-8)
ELR-CXC chemokine
G31P
TNF,IL-1
C5a,LTB4,PAF
C5a
TNF, IL-1
AM
Epithelial cells
G31P/CXCR1
G31P
3
?
IL-8
C5a,LTB4,
PAF gradient
IL-8
G31P
Other type of cells
2
TNF, IL-1, IL-8
ROI release, Ca2+ flux,
delayed apoptosis
G31P
4
?
IL-8
CXCR1/2/G31P
IL-8, LTB4, PAF
I/R
C5a
1
G31P
GAG&PAF
GAG& IL-8
GAG&G31P
CXCR1/2
C5aR,BLT,FPR
PMN
β2 integrin
Endothelial cells

Like repertaxin, G31P may be also useful in treating sepsis, an important contributor for ARDS, if given at optimal times via s.c. or i.p. routes. It is well established that CXCR2 expression on neutrophils is down-regulated in sepsis patients, which seems to indicate that CXCR2 ligands play a minor role in mediating neutrophil migration. But CXCR1 is normally expressed and IL-8 still can induce chemotactic responses in neutrophils from ARDS patients (Cummings, Martin et al. 1999). Recently study found that neutralization of CXCR2 before CLP surgery significantly protected mice against CLP-induced mortality. CXCR2 knockout mice were also protected against CLP-induced mortality compared with wild type mice. However, blocking CXCR2 after the CLP procedures was not beneficial to survival (Ness, Hogaboam et al. 2003), which indicates that blocking CXCR2 is only effective before CXCR2 signaling leading to cellular activation. In other words, when neutrophils are already activated during sepsis, blockade of CXCR2 alone may not be enough to protect against mortality in sepsis. Therefore, as a CXCR1 and CXCR2 antagonist, G31P would be effective in blocking neutrophil infiltration into the peritoneal cavity and decrease mortality in the CLP model if given before and after CLP surgery. It may be also effective after sepsis is already developed given that it also blocks CXCR1 signaling on neutrophils. In addition, C5a- and fMLP-induced neutrophil activation is also important in sepsis pathology (Czermak, Sarma et al. 1999). The combined effects of G31P on the CXCR1 and CXCR2 and the alternate receptors for C5a and fMLP may contribute to inhibit neutrophil activation and migration and reduce pathology in sepis patients. But these combined effects of G31P may not be effective in treating those sepsis patients wherein their neutrophils have already been fully activated. These speculations need to be further examined.

G31P may not be beneficial in some infections. One study found that CXCR2 is important in protection from some bacterial infection, such as from *Pseudomonas aeruginosa*. Blockade of ELR-CXC chemokines can only modestly reduce neutrophil infiltration and has no effect on bacterial clearance or survival. Blockade of CXCR2 resulted in a marked increase in mortality, which was associated with dramatic decreases in neutrophil recruitment and bacterial clearance (Tsai, Strieter et al. 2000). Similarly, CXCR2 also has protective functions in *Aspergillus fumigatus* infections (Mehrad, Strieter et al. 1999). Therefore, G31P treatment may be not beneficial in these settings. The

reason may be because ELR-CXC chemokines and their receptors are less important for activating neutrophils, but necessary and protective in severe infections. This is also suggested in the finding that fMLP seems to be important in situations with high bacterial inocula in inducing neutrophil migration and activation, whereas chemokines are important in low bacterial inoculum situations and contribute to more sustained neutrophil responses (Gauthier, Fortin et al. 2007).

As discussed above, G31P perhaps would be effective in treating ALI/ARDS patients. It may be beneficial in treating neutrophil-mediated chronic diseases, such as RA and COPD, which may need long-term treatment. This brings the question whether using G31P for long-term treatment will cause any adverse effects for the body. It has been indicated that anti-TNF antibody treatment can cause malignacies and serious infections (Bongartz, Sutton et al. 2006). It may also increase the risk of developing some other diseases, such as tuberculosis, neurological disease, autoimmune disease, and cardiac disease (Hyrich, Silman et al. 2004). There were concerns about the risk of these diseases when using anti-TNF treatment because TNFα has a crucial role in the body's defense against foreign pathogens such as bacteria, viruses, and fungi, particularly in the recruitment of neutrophils and monocytes to the site of infection. Similarly, G31P treatment via binding CXCR1 and CXCR2 has a potent effect on blocking neutrophil recruitment. The question that may be brought here is whether G31P treatment will increase the risk of serious infection. Our results suggest that bacterial growth did not increase after treatment with G31P in an aspiration pneumonia model, but the effect of its long-term use will need to be investigated critically. In addition, CXCR2 is protective during infection by some microorganisms, such as *Pseudomonas aeruginosa* and *Aspergillus fumigatus* (Mehrad, Strieter et al. 1999; Tsai, Strieter et al. 2000). Blockade of the CXCR2 using G31P may increase the risk of infection by these kinds of pathogens.

General conclusion:

We successfully developed a human ELR-CXC chemokine antagonist, human $CXCL8_{(3\text{-}72)}$K11R/G31P (hG31P). It can effectively block neutrophil chemotactic responses and activation induced by ELR-CXC chemokines and othe neutrophil

chemoattractants *in vitro*. It also effectively blocks neutrophil recruitment and activation, increased vascular permeability, and cytokine and chemokine expression in *in vivo* systems, including experimental models of airway endotoxemia, aspiration pneumonia, and mesenteric I/R injury. hG31P could potentially be a viable therapeutic approach for inflammatory diseases such as ALI/ARDS.

Future directions

We have shown that G31P effectively blocks neutrophil chemotaxis and calcium flux responses to ELR-CXC chemokine, including CXCL8 and CXCR2-exclusive chemokines (e.g., CXCL1, CXCL5), and ROI release stimulated by CXCL8, which indicates that G31P can competively bind CXCR1 and CXCR2 on neutrophils. But it would be ideal to have direct evidence regarding the affinity of G31P for the CXCR1 and CXCR2, relative to IL-8, GRO, or GCP-2.

Neutrophil chemoattractants employ different intracellular signaling pathways, wherein IL-8, PAF, and LTB_4 are dependent on PI3K, whereas p38 MAK-dependent signaling mediates neutrophil responses to fMLP and C5a. There is a need to know how G31P binding to the CXCR1 or CXCR2, or both receptors, heterologously desensitizes alternative ligand receptors. What other pathways does G31P blunt and possibly employ to effect heterologous desensitization of receptors for C5a and fMLP? Elucidating these details will help us further understand the mechanism of how G31P works *in vivo*.

There is a similar need for studies on how G31P affect LPS challenged-epithelial cell activation and cytokine and chemokine expression. As discussed in the literature review (§1.2.1.4), LPS activation of neutrophils through TLR4 can down-regulate the CXCR1 and CXCR2 expression, although CXCR1 expression is more resistant to the down-regulation. It would be interesting to know whether G31P binding to CXCR1 or CXCR2 negatively affects TLR4 expression, resulting in decreased cytokine and chemokine expression by epithelial cells or if this is accomplished by some other mechanism. Also, it will be interesting to determine whether G31P can block LPS-induced cytokines and chemokines expression from neutrophils and endothelial cells. If so, what would be the operative mechanisms?

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